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Inorganic Nitrogen Assimilation by *Micrococcus sodonensis*

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis, entitled "Inorganic Nitrogen Assimilation by Micrococcus sodonensis", submitted by James N. Bunch, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

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Abstract

Micrococcus sodonensis was found to be dependent upon ammonium for growth. During the logarithmic growth phase of a culture, the amount of NH_4^+ in the medium decreased by 32.2%. In the absence of NH_4^+, growth proceeded at a much slower rate.

The activity of glutamic acid dehydrogenase was ascertained in this organism. Campbell, Evans, Perry and Niven (1961) found large quantities of α-keto-glutarate evolved in a culture in the absence of NH_4^+. NH_4^+ and the amino group of glutamic acid have been shown to enter into the synthesis of guanosine-5'-monophosphate via xanthosine-5'-monophosphate aminase, which is active in this organism.

The activity of carbamyl phosphate synthetase was demonstrated by reversing the direction of the reaction. ATP was produced from carbamyl phosphate and ADP.

Ornithine transcarbamylase and aspartic acid transcarbamylase, two enzyme systems specific for carbamyl phosphate, were demonstrated. The products of these systems are citrulline and carbamyl-aspartic acid, respectively. Carbamyl-aspartic acid is a precursor of uridine-5'-monophosphate and cytidine-5'-monophosphate.

The substitution for NH_4^+ in the growth medium of guanosine, and to a lesser extent, cytidine and uridine, resulted in a duplication of the NH_4^+ effect.

Cultures of Micrococcus sodonensis were enriched with

$N^{15}H_4Cl$. After 30 seconds, a level of enrichment at 0.9 At.% N^{15} was established in the early nucleotide pool. The constituents of the pool were found to be 5' nucleotides.

After 20 minutes, a level of enrichment at 6.5 At.% N^{15} was established in the amino acid pool. The pool was found to contain glutamic acid, aspartic acid, ornithine, alanine, valine or methionine, and leucine or isoleucine.

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List of Abbreviations

aap	amino acid pool
ADP	adenosine triphosphate
ala	alanine
⬡-ala	phenylalanine
AMP	adenosine-5'-monophosphate
Ap	3' adenylic acid
Aq.	aqueous
arg	arginine
asp	aspartic acid
At.%	atom percent
ATP	adenosine triphosphate
CAP	carbamyl phosphate
cap	early nucleotide pool (cold acid pool)
CMP	cytidine-5'-monophosphate
Cp	3' cytidylic acid
CV	column volume
dA	deoxyadenosine
dC	deoxycytidine
dG	deoxyguanosine
dN	deoxynucleoside
dT	deoxythymidine
DNA	deoxyribonucleic acid
DPA	diphenylamine
EDTA	ethylene diaminotetraacetic acid
glu	glutamic acid
gly	glycine
G-6-P deHase	Glucose-6-Phosphate dehydrogenase

GMP	guanosine-5'-monophosphate
Gp	3' guanylic acid
hist	histidine
isoleu	isoleucine
leu	leucine
ma	milliamperes
mv	millivolts
$NADP^+$	nicotinamide adenine dinucleotide
Np	3' nucleotide
OD	optical density
orn	ornithine
pA	5' adenylic acid
pC	5' cytidylic acid
pdA	5' deoxyadenylic acid
pdC	5' deoxycytidylic acid
pdG	5' deoxyguanylic acid
pG	5' guanylic acid
pN	5' nucleotide
T	thymidine
TCA	trichloroacetic acid
tryp	tryptophane
T.S. broth	trypticase soy broth
tyr	tyrosine
μM	micromoles
UMP	uridine-5'-monophosphate
Up	3' uridylic acid
UV	ultraviolet
XMP	xanthosine-5'- monophosphate

Pi	inorganic phosphate
PPi	pyrophosphate
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

INTRODUCTION

Ammonium plays a major role in nitrogen metabolism in microorganisms. In addition to serving as a sole source of nitrogen for many forms, it is probably an intermediate in the assimilatory reactions of other nitrogen compounds, ie. NO_3, NO_2, N_2. Ammonium is considered by many to be the ultimate form in which all inorganic nitrogen compounds are finally incorporated into the organic fraction of the cell. This study deals with the role of NH_4^+ in the metabolism of a heterotrophic organism which will not use it as a sole source of nitrogen.

Several systems for the incorporation of NH_4^+ exist in nature (Mortenson - 1962).

1. Amidation

The incorporation of NH_4^+ into amide groups occurs by means of the following reaction:

$$R\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH + NH_4^+ \rightleftharpoons R\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH_2$$

Glutamine is a major product of NH_4^+ incorporation in both plants and animals, and in some, is a key amino donor. The synthesis of this compound has been demonstrated in cell-free preparations of Staphylococcus aureus by Elliot and Gale (1948), and proceeds according to the following sequence:

$$\text{glutamic acid} + NH_4^+ + ATP \xrightleftharpoons[\text{glutamine synthetase}]{Mg^{++}} \text{glutamine} + ADP + Pi$$

The requirements of the reaction are the same in plant, animal and bacterial tissues.

The amidation of aspartic acid results in the formation of asparagine. This reaction has not been demonstrated in bacteria, but in wheat germ, a system similar to glutamine was found by Webster and Vainer (1955). C^{14}-labelled aspartic acid was converted to asparagine, with NH_4^+ incorporated into the amide group. An alternative exists, however, with asparagine synthesis resulting from ω-transamidation from glutamine to aspartic acid.

2. Amination

The incorporation of NH_4^+ into α-keto acids as an α-amino group occurs by means of the following reaction:

$$R\text{-}\overset{\overset{O}{\|}}{C}\text{-}COOH + NH_4^+ \rightleftharpoons R\text{-}\overset{\overset{NH_2}{|}}{CH}\text{-}COOH$$

Glutamic acid dehydrogenase appears to be almost universal in its distribution. Its presence has been noted in animal and plant cells and in yeast, bacteria and fungi. Cell-free preparations of Escherichia coli have been demonstrated by Adler, Hellstrom, Gunther and von Euler (1938) to deaminate glutamic acid in two steps:

$$\text{L-glutamic acid} + NADP^+ \rightleftharpoons \text{iminoglutaric acid} + NADPH.H^+$$

$$\text{iminoglutaric acid} + HOH \rightleftharpoons \alpha\text{-keto-glutaric acid} + NH_4^+$$

The reaction is specific for L-glutamic acid and $NADP^+$ and is freely reversible, linking it to the Kreb's cycle. Kun, Ayling, and Baltimore (1964), employing selective inhibitors, have demonstrated a sequential enzymatic mechanism that involves an

obligatory coupling of glutamic acid dehydrogenase and glutamate-aspartate and glutamate-alanine transaminases in kidney tissue of rats.

The glutamic acid dehydrogenase system was, at one time, considered to be the essential mechanism for the incorporation of NH_4^+ into organic compounds. However, other dehydrogenases have since been found to play a role. For example, Wiame and Pierard (1955) have demonstrated a specific alanine dehydrogenase in a mutant of Bacillus subtilis.

The aspartase system was first demonstrated by Quastel and Woolf (1926) in E. coli.

$$\text{L-aspartic acid} \xrightleftharpoons{\text{aspartase}} \text{fumaric acid} + NH_4^+$$

The enzyme is specific for L-aspartic acid and fumaric acid and is freely reversible. Fumaric acid formed by the deamination of aspartic acid is rapidly reduced to succinic acid. It has been suggested that this enzyme system has little or no biosynthetic function.

3. Purine Synthesis

The synthesis of guanosine-5'-monophosphate (GMP) from xanthosine-5'-monophosphate (XMP) has been demonstrated, in Aerobacter aerogenes by Lieberman (1956), and by Moyed and Magasanik (1957) in E. coli, to be accomplished by an enzyme specific for XMP and NH_4^+.

$$XMP + NH_4^+ + ATP \xrightarrow[\text{XMP aminase}]{Mg^{++}} GMP + AMP + PPi$$

While NH_4^+ appears to be the exclusive amino donor in bacteria, Abrams and Bentley (1959) have reported glutamine to be the amino donor in animal cells. The sequence is not reversible in either case.

4. Carbamyl Phosphate Synthesis

The synthesis of carbamyl phosphate (CAP) from ammonium carbonate and ATP was demonstrated in a cell-free preparation of Streptococcus faecalis by Jones, Spector and Lipmann (1955).

$$CO_2 + NH_4^+ + ATP \rightleftharpoons CAP + ADP$$

Ravel, Humphreys and Shive (1961) obtained the purified enzyme from a cell-free preparation of Streptococcus lactis and found ammonium carbamate to be a more effective substrate for CAP production. The rate of CAP synthesis was increased by coupling it with the carbamylation of ornithine to yield citrulline.

Synthesis of CAP involves carbamate kinase and carbamyl phosphokinase and is freely reversible as evidenced by the phosphorylation of ADP.

$$NH_4^+ + CO_3^= \underset{\text{kinase}}{\overset{\text{carbamate}}{\rightleftharpoons}} H_2N\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}NH_2$$

$$H_2N\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}NH_2 + ATP \underset{\text{phosphokinase}}{\overset{\text{carbamyl}}{\rightleftharpoons}} CAP + ADP$$

The existence of carbamate kinase has not been definitely established, and Jones and Lipmann (1960) note the possibility of carbamate formation being a spontaneous reaction of carbonate and NH_4^+.

In contrast to this system, Fahien and Cohen (1963), working with frog liver tissue, have demonstrated a single enzyme system - CAP synthetase. This system requires N-acetyl glutamate as a cofactor and two moles of ATP per mole of CAP produced. The reaction proceeds in two steps:

$$ATP + HCO_3^- \xrightarrow[\text{acetylglutamate}]{Mg^{++}} ADP + Pi + \text{"active" } CO_2$$

$$ATP + \text{"active" } CO_2 + NH_4^+ \xrightarrow[\text{glutamate}]{\text{acetyl-}} CAP + ADP$$

The reaction is only partially reversible. Evidence has been presented that the enzyme possesses two binding sites for ATP, one which is independent of other substrates and one which requires prior binding of N-acetylglutamate.

CAP plays an integral role in the formation of citrulline and carbamyl-aspartic acid (ureidosuccinic acid), which in turn, are essential for the synthesis of arginine and uridine-5'-monophosphate.

In the synthesis of citrulline, the enzymatic reaction is catalysed by ornithine transcarbamylase. Reichard (1957) reported the reaction to be reversible and specific for L-ornithine in rat liver.

$$\text{L-ornithine} + CAP \underset{\text{transcarbamylase}}{\overset{\text{ornithine}}{\rightleftharpoons}} \text{citrulline} + H^+$$

Arginine may then be synthesized from citrulline and the NH_4^+ incorporated ultimately enters into protein synthesis.

The carbamylation of aspartic acid forms carbamyl-aspartic acid and is catalysed by aspartic acid transcarbamyl-

ase. This reaction was first seen by Reichard in rat liver mitochondria (1954). Carbamyl-aspartic acid is an intermediate in the synthesis of pyrimidines.

The actual mode of uptake of NH_4^+ has been suggested by Zarlengo and Abrams (1963) to be simple non-ionic diffusion in Streptococcus faecalis. Cells with a low intracellular pH were retarded in glycolysis but this was restored by the addition of NH_4^+ to the medium. The rise in intracellular pH was accompanied by an increase in extracellular hydrogen ion (H^+) concentration. This was measured by continuous titration of the medium with alkali to maintain the initial pH of 7. Centrifugation of the cells and measurement of NH_4^+ content with Nessler's reagent showed the loss of NH_4^+ from the medium to be equivalent to the appearance of H^+ ions.

A process of non-ionic diffusion of NH_4^+ was substantiated by suspending cells in a solution of NH_4Cl at pH 7 and measuring the uptake of NH_4^+. These cells were then washed, suspended in water, and the release of NH_4^+ was measured by titration with HCl.

In the case of Micrococcus sodonensis, it was noted by Campbell, Evans, Perry and Niven (1961) that a specific requirment for NH_4^+ existed when the organism was grown in a synthetic medium. In the absence of NH_4^+, large quantities of α-keto-glutarate were evolved and released into the medium. The effect was not duplicated by substituting K^+ or Na^+ for NH_4^+. Excess glutamic acid in the medium did not prevent the accumulation of α-keto-glutarate.

It was hypothesized that NH_4^+ was required for something

other than glutamic acid biosynthesis and that the NH_4^+ deficiency was satisfied by deaminating glutamic acid and releasing the surplus keto acid formed. The requirement for NH_4^+ was suggested to lie in the synthesis of pyrimidines via carbamyl phosphate.

In addition to the activity of various enzymes, a major portion of the present investigation was concerned with isotope studies. N^{15}, the heavy nitrogen isotope, is being employed in ever-expanding fields of study. The unstable nitrogen isotope, N^{13}, has a half-life of only 9.93 minutes, and is, therefore, unsuitable to many biological investigations, although it has been used.

Sprinson and Rittenberg (1948-1949) employed N^{15} in their investigations of protein metabolism in mammalian tissue. Many of the techniques involved in the use of N^{15} in biological systems are described in their papers.

Much has been learned about nitrogen fixation through isotope studies. In 1952, Aprison and Burris noted the rate and mechanism of nitrogen fixation in soybean nodules by means of the heavy isotope. Bergersen (1960) fractionated soybean nodules and determined the incorporation of N^{15} into the various fractions.

The fate of nitrogen fertilizer in soils has been investigated using N^{15}. Martin, Henzell, Ross and Haydock (1963) measured isotope enrichment in the soil, and in the plants growing in the soil, to determine the extent of fertilizer loss. Errors encountered during chemical and mass spectrometric analysis of the soil and plant material were discussed in their

paper.

Organic nitrogen samples are converted to ammonium sulphate by means of Kjeldahl digestions. The salts are oxidized to nitrogen gas (N_2) in a vacuum system, and the gas is analysed in a mass spectrometer for masses 28 ($N^{14}N^{14}$) and 29 ($N^{14}N^{15}$). Errors in these analyses may result from several factors, all of which must be taken into consideration.

Free or combined natural sources of nitrogen contain an approximately constant fraction of the heavy isotope although minor variations have been reported. The use of N^{15} as a tracer requires the correct determination of N^{15} content in excess of the natural abundance, hence in gas samples containing low enrichment, quite large proportional errors can be introduced by incorrect estimations of natural abundance. In the present investigation natural abundance was measured in non-enriched controls.

The use of the same Kjeldahl apparatus for repeated isotope samples can result in a carry-over of the isotope. That is, traces of a previous sample may contaminate the next sample. This is particularly evident when there is extreme variation in the isotopic enrichment of the samples. This is termed a memory effect, and can be minimized but not eliminated by proper cleaning of the apparatus between successive samples. Memory effects in the mass spectrometer itself are negligible.

Calculations of natural abundance of N^{15} show that they are inversely related to sample pressure in the mass spectrometer. Martin et al obtained values ranging from 0.365 (using

the maximum charge of gas) to 0.401 At. % N^{15} (at a gas pressure of 2% of the maximum). As large a gas volume as is practicable is, therefore, always used.

Sources of contamination such as atmospheric gases, oxygen evolved from the hypobromite solution, water vapour and ethanolic indicators can all contribute to the size of masses 28 and 29 (see Part II - Experimental) and these errors must be dealt with in any heavy nitrogen experiment.

Some idea of the variability arising at different stages in the analyses of Martin et al was obtained from replicated isotopic measurements of selected samples.

Instrumental errors include reproducibility in potentiometer readings, isotopic fractionation at the inlet leak in the ion source, and background variations, all of which were too small to influence the results significantly.

Martin et al found the effect of transferring the gas to a breakseal tube, which involves manipulation of a gas-handling system, was to increase the co-efficient of variation sixfold to 0.21% at this stage, probably since air contamination of gas samples can occur during this step.

Variations between duplicate solutions, obtained from the same Kjeldahl distillate were larger than expected and may have been influenced by small memory effects or traces of ethanol. Variation between duplicate soil samples showed a further increase in variability of the data, but the co-efficient of variation obtained (1.53%) was considered satisfactorily small.

With all of these sources of error taken into account,

reproducible results in the present investigation were considered accurate, and unpredicted low values were assumed to be a result of contamination.

The purpose of this investigation was to examine the route(s) of NH_4^+ incorporation in Micrococcus sodonensis and to see if systems other than glutamic acid dehydrogenase were involved. Evidence for the incorporation of NH_4^+ into nucleic acid biosynthesis by way of amination of XMP and carbamyl phosphate synthesis, as well as into protein biosynthesis, again by way of CAP synthesis, will be presented.

PART I

Nutritional and Enzymatic Studies

The initial investigation into the nitrogen metabolism of Micrococcus sodonensis involved a study of the effect on growth of possible products of NH_4^+ incorporation, other than glutamic acid, in the presence and absence of NH_4^+. With the dependence of the organism upon NH_4^+ established, the various routes of its incorporation were determined.

PART I

Materials and Methods

1. Organism and Culture Conditions

The organism used throughout this investigation was Micrococcus sodonensis (ATCC 11880). The synthetic medium employed was that of Campbell, Evans, Perry and Niven (1961) and was prepared as follows:

$CaCl_2$	1.0 mg
$Na_6Mo_7O_{24}$	80.0 µg
$ZnSO_4$	1.0 mg
$MnSO_4$	0.8 mg
H_3Bo_3	0.4 mg
$CuSO_4$	0.1 mg
CoCl	0.1 mg
E.D.T.A. (Versene)	50.0 µg
$MgSO_4 \cdot 7H_2O$	20.0 mg
KCl	50.0 mg
$FeSO_4$	0.4 mg
NH_4Cl	50.0 mg
sodium glycerol phosphate	10.0 mg
biotin	1.0 µg
lactic acid	500.0 mg
glutamic acid	500.0 mg

Tris buffer (Fisher Scientific) was added to yield a final concentration of M/30 and the pH was adjusted to 7.3 with M NaOH. The volume was adjusted to 1000 ml. with distilled

water. The medium was normally made up in 500 ml. quantities and dispensed into 50 ml. Erlenmeyer flasks in 20 ml. aliquots. These were sterilized by autoclaving, and stored at 6°C. until used.

Trypticase soy broth (T.S.broth) was prepared according to the manufacturer's directions (Baltimore Biological Laboratories).

Cultures were maintained on T.S. broth plus 1.5% agar (Difco). When liquid cultures were required, cells were incubated in T.S. broth or synthetic medium. Incubation was at room temperature, with continuous agitation on a mechanical shaker.

Cultures were prepared for storage by adding one drop of a 24 hour culture of cells to 5 ml. aliquots of T.S. broth dispensed in 8 ml. vials. The suspensions were stored at -50°C.

Inocula were prepared as follows: cells of a 24 hour culture were washed twice and resuspended in sterile distilled water to yield an optical density (OD) of 1.0 at 600 mu as measured in a Bausch and Lomb Spectronic 20 colourimeter. Of this preparation, 0.1 ml. per 20 ml. of culture medium was used as the inoculum.

The growth response of Micrococcus sodonensis to various energy sources was determined by substituting these for lactic acid in the synthetic medium to form a final concentration of 0.55 μM/ml. Inulin and dextrin, which do not have any specific single molecular weight value, were judged to be twice as heavy as a triose and a solution was made accordingly.

The response to nucleic acid derivatives was determined

by substituting these for NH_4^+ in the synthetic medium to yield a final concentration of 1.0 μM/ml.

In all cases, growth response was measured turbidimetrically at 600 mμ in a Bausch and Lomb Spectronic 20 colourimeter. These readings were converted to mg. dry weight of cells by means of a previously prepared standard curve of OD vs. dry weight of washed cells (see Appendix - Figure 14).

Cell-free suspensions were obtained by centrifuging 40 ml. of a 24 hour culture grown in synthetic medium. The cells were washed 3 times with 0.07 M phosphate buffer at pH 7 and then frozen as a solid plug. The plug was put into a Hughes press cooled to -50°C, and 12 tons pressure were applied, after which the disrupted cells were removed and suspended in 0.07 M phosphate buffer at pH 7. The suspension was centrifuged at 10,000 rpm for 5 minutes to remove whole cells.

2. Chemical Assays

(a) Ammonium

The detection and quantitation of NH_4^+ was accomplished by means of the Nessler's reaction (Collowick and Kaplan - 1957). To each 1.0 ml. sample was added 1.0 ml. of Nessler's reagent (Fisher Scientific) and 1.0 ml. of 0.4% aq. gum arabic. This mixture was brought to a final volume of 10 ml. with distilled water. After a 5 minute period of colour development, the OD's of the solutions were measured colourimetrically at 400 mμ. These readings were then converted to μM of NH_4^+ by means of a previously prepared standard curve obtained by measuring the OD's of known concentrations of ammonium chloride solutions

developed by the above procedure, and plotting NH_4^+ concentration (μM/ml.) against OD (see Appendix - Figure 15).

(b) α-keto-glutaric acid

The electrophoretic technique of Berry and Campbell (1964) was employed for the determination of α-keto-glutarate, formed through the activity of the glutamic dehydrogenase system.

Electrophoresis was carried out using the LKB paper electrophoresis apparatus No. 3276B and 40x410 mm. Schleicher and Schuell filter paper strips. Volumes of the sample (0.1 ml.) were streaked across the paper strip which was impregnated with 0.125 M Veronal buffer at pH 8.6. The streak was allowed to run for a period of 4 hours with a current of 10 ma and 200 mv at room temperature (25°C). After drying, the strips were sprayed with 0.05% 2,4-dinitrophenylhydrazine in 2 N HCl. After a 5 minute period of colour development, the strips were examined for yellow spots which represented the 2,4-dinitrophenylhydrazone derivatives of the α-keto acids. (Veronal - see appendix).

(c) Citrulline

The determination and quantitation of citrulline was accomplished by means of the technique of Archibald (1944). To 0.5 ml. of reaction mixture was added 3.5 ml. of distilled water, 1.0 ml. of acid reagent (1 volume cH_2SO_4 - 3 volumes cH_3PO_4), and 0.25 ml. of 3% aq. diacetyl monoxime. The mixtures were sealed in screwcap vials which were wrapped in foil and placed in a boiling water bath for ten minutes. The solutions were then cooled to room temperature for ten minutes and their OD's at 490 mμ measured in a Bausch and Lomb Spectronic 20 colourimeter. The readings were converted to

μM of citrulline per ml. of solution by means of a previously prepared standard curve (see Appendix - Figure 16).

The standard curve was prepared by using the same procedure with known solutions of citrulline. OD values at 490 mμ were plotted against the concentrations of citrulline. The curve was linear only between citrulline concentrations of 0 to 0.3 μM. All subsequent experimental samples were diluted to fall within this range.

(d) Carbamyl-aspartic Acid

The procedure of Koritz and Cohen (1954) was used to detect and quantitate carbamyl-aspartic acid (ureidosuccinic acid).

To a 0.5 ml. portion of a reaction mixture was added 2.5 ml. of distilled water, 6.0 ml. of 50% v/v cH_2SO_4, 0.1 ml. of aq. sodium diphenylamine-p-sulfonate (sulfonic acid reagent), and 0.25 ml. of 3% aq. diacetyl monoxime. The solutions were mixed and capped with corks vented by means of a capillary tube. They were then placed in a boiling water bath for ten minutes, cooled, and 0.25 ml. of 1% $K_2S_2O_8$ was added. The tubes were recapped, wrapped in foil and placed in a boiling water bath for 1 minute. The OD's, at 550 mμ, of the solutions were read after cooling for 10 minutes. The OD's were then converted to μM of carbamyl-aspartic acid by means of a previously prepared standard curve. (see Appendix - Figure 17).

The standard curve was prepared by plotting known values of carbamyl-aspartic acid standards against the values obtained by the above procedure and determining the colour development by reading the OD's at 590 mμ.

(e) Orthophosphate

The procedure of Fiske and SubbaRow (1925) was employed to determine orthophosphate in reaction mixtures. One ml. samples were deproteinized by the addition of 1.0 ml. of cold 5% TCA. After centrifugation, the supernatant was divided into two portions and the orthophosphate content measured before and after hydrolysis with NaOH.

One ml. of 2.5% $(NH_4)_6Mo_7O_{24}$ in 3N H_2SO_4 was added to 0.5 ml. of reaction mixture, after which 0.4 ml. of 0.25% aminonaphtholsulfonic acid was added. The mixture was brought to a final volume of 10 ml. All steps were carried out at 0^oC. After 10 minutes, colour development was measured on a Spectronic 20 colourimeter at 660 mμ. The values obtained were converted to μM/ml. of orthophosphate by means of a previously prepared standard curve (see Appendix - Figure 18).

Hydrolysis of metaphosphate (see above) was accomplished by mixing the deproteinized sample with an equal volume of 0.1N NaOH, and leaving the mixture for 10 minutes at 25^oC. The analysis for orthophosphate was then carried out.

The standard curve for orthophosphate was prepared by analysing standard solutions of orthophosphate by the above procedure and plotting OD values obtained against the known concentrations of the solutions.

(f) Mononucleotides

Mononucleotides were isolated and quantitated according to the procedure of Singh and Lane (1963). A 24 inch strip of Whatman's No. 1 filter paper was dipped in a saturated solution of $(NH_4)_2SO_4$ and left to dry. Two hundred lambda of

the solution to be tested were applied to the paper which was then developed for 18 hours at room temperature in a solvent system composed of 95% CH_3CH_2OH and distilled HOH (80:20).

At the end of that time, the spots were located with a UV handlamp, cut out in standard squares (3 1/2 x 2 1/2 inches) and eluted in 4 ml. of distilled water overnight. The OD's at 260 mμ, of the eluents, were then measured on a Beckman model DU spectrophotometer, and converted to μM of nucleotide by means of a previously prepared standard curve (see Appendix - Figure 19) which was obtained by plotting OD's at 260 mμ against known concentrations of control nucleotides treated chromatographically as described.

3. Enzymatic Assays

(a) Glutamic Acid Dehydrogenase Activity

The ability of resting cells to deaminate glutamic acid in the presence of lactic acid, NH_4Cl and various combinations of these was demonstrated by means of the Conway microdiffusion technique (Conway-1947).

The inoculum consisted of a suspension of washed cells (24 hour culture) at OD 1, in a 0.07M phosphate buffer at pH 7. Stock solutions were adjusted to pH 7 with NaOH and HCl and included:

NH_4Cl	25 μM/ml.
lactic acid	25 μM/ml.
glutamic acid	25 μM/ml.
phosphate buffer (pH 7)	0.07M
N-saline	

The materials were added to the outer wells of 66 mm. Conway diffusion plates according to the following protocol. N-saline was substituted for the inoculum in a duplicate control for each reaction plate.

(Volume in ml.)

Plate	Buffer	cells / N-saline	NH_4Cl	glu $\bar{a}$	lact $\bar{a}$
cells + buffer	1.5	0.5	0.0	0.0	0.0
NH_4Cl	1.1	0.5	0.4	0.0	0.0
glutamic acid	1.1	0.5	0.0	0.4	0.0
lactic acid	1.1	0.5	0.0	0.0	0.4
lactic acid + glutamic acid	0.7	0.5	0.0	0.4	0.4
lactic acid + NH_4Cl	0.7	0.5	0.4	0.0	0.4
lactic acid + glutamic acid + NH_4Cl	0.3	0.5	0.4	0.4	0.4

In all cases, the final volume in the outer wells was 2.0 ml. and pH 7. The inoculum was added last.

The inner well of each plate contained 1.0 ml. of 0.1N H_2SO_4.

The plates were sealed and incubated at 30°C. for designated periods, after which 1 ml. of saturated K_2SO_4 was added to the culture in the outer well of each plate, which was immediately sealed again. The plates were then incubated for a further two hours at 30°C. in all cases. At the end of this time 0.25 ml. of H_2SO_4 was drawn from the centre well and

assayed for NH_4^+ by means of the Nessler's reaction.

(b) Ornithine Transcarbamylase Activity (Archibald 1944)

The citrulline assay technique of Archibald allowed a quantitative estimation of the activity of ornithine transcarbamylase.

The materials employed in the reaction included:

Tris buffer pH 8.5	200 µM/0.2 ml.
ornithine	10 µM/0.2 ml.
$MgCl_2$	5 µM/0.2 ml.
CAP	6.2 µM/0.2 ml.
disrupted cell inoculum	0.2 ml.

The disrupted cell preparation was prepared immediately prior to the experiment. The materials were added together in 10 ml. Erlenmeyer flasks according to the following protocol:

Materials:	(Volume in ml.)				
Tube	1	2	3	4	5
Tris	0.2	0.2	0.2	0.2	0.2
ornithine	0.2	0.2	0.0	0.2	0.0
$MgCl_2$	0.2	0.2	0.2	0.2	0.0
CAP	0.2	0.2	0.2	0.0	0.0
disrupted cell preparation	0.2	0.0	0.2	0.2	0.2
HOH	0.0	0.2	0.2	0.2	0.6

The cell-free preparation was added last. The final volume in all cases was 1.0 ml.

After 30 minutes incubation in a water bath, 0.5 ml. of

the reaction mixture was drawn off and added to 3.5 ml. of distilled water and tested for the presence of citrulline.

(c) Aspartic Acid Transcarbamylase Activity

The carbamyl-aspartic acid assay of Koritz and Cohen (1954) was used to quantitate the activity of aspartic acid transcarbamylase. The following materials were employed:

1.0M Tris buffer pH 8.5

0.1M CAP

0.2M L-aspartic acid

disrupted cell inoculum

The materials were added together in 10 ml. flasks as designated in the following protocol:

Materials	(Volume in ml.)				
Tube	1	2	3	4	5
Tris	0.2	0.2	0.2	0.2	0.2
CAP	0.2	0.2	0.0	0.2	0.0
L-aspartic acid	0.2	0.2	0.2	0.0	0.0
HOH	0.2	0.4	0.4	0.4	0.6
disrupted cell preparation	0.2	0.0	0.2	0.2	0.2

In all cases, final volume was 1.0 ml. and pH 8.5. The disrupted cell inoculum was added last.

After 30 minutes incubation in a water bath at 37°C, 0.5 ml. was drawn off and assayed for carbamyl-aspartic acid according to the procedure of Koritz and Cohen. (1954)

(d) Carbamyl Phosphate Synthetase Activity

Several methods were employed for the detection of this enzyme system.

i. Substituted for CAP in the ornithine transcarbamylase reaction were:

250 μM NH_4Cl

125 μM $NaHCO_3$

125 μM Na_2CO_3

8 μM ATP

The procedure for the detection of citrulline was then followed.

ii. Aspartic acid was substituted for ornithine in the above system (i) and the procedure for the detection of carbamyl-aspartic acid was then followed.

iii. A direct measurement of CAP was possible by employing the procedure of Fiske and SubbaRow for the assay of orthophosphate. The enzyme reaction utilized the following:

10 μM $MgCl_2$

50 μM Tris buffer pH 8.5

250 μM NH_4Cl

125 μM $NaHCO_3$

125 μM Na_2CO_3

8 μM ATP

disrupted cell inoculum

The materials were added together to form a 1.0 ml. volume which was incubated in a water bath for 30 minutes at 37°C., and then deproteinized by the addition of 1.0 ml. of cold 5% TCA. The sample was divided into two portions and the ortho-

phosphate content measured before and after hydrolysis with NaOH by means of the Fiske and SubbaRow procedure. The difference in orthophosphate content between the two portions was the CAP content before hydrolysis.

iv. The activity of CAP synthetase was determined by reversing the equilibrium of the enzyme system and assaying for the formation of ATP. The reaction mixture included the following:

0.02M ADP

0.02M ATP

0.1M CAP

60.0 μg hexokinase

50.0 μg glucose-6-phosphate dehydrogenase

5.0 μM glucose

0.002M $NADP^+$

30.0 μM $MgCl_2$

0.1M Tris buffer pH 8.5

disrupted cell inoculum

The materials were added together in quartz curvettes according to the following protocol:

Materials		(Volumes in ml.)			
	Tube	1	2	3,4	5,6
	ADP	0.0	0.0	0.2	0.2
I	CAP	0.0	0.0	0.0	0.2
	inoculum	0.0	0.0	0.5	0.5
	ATP	0.0	0.5	0.0	0.0
	glucose	0.2	0.2	0.2	0.2
II	hexokinase	0.5	0.5	0.5	0.5
	$NADP^+$	0.5	0.5	0.5	0.5
III	G-6-P deHase	0.5	0.5	0.5	0.5
	$MgCl_2$	0.2	0.2	0.2	0.2
	Tris	0.2	0.2	0.2	0.2
	HOH	0.9	0.4	0.2	0.0

The final volume in all cases was 3.0 ml.

In all cases, the G-6-P deHase and $NADP^+$ (enzyme system I) were added after five minutes incubation of the other components, in order to bypass any lag in the reaction. In the case of tubes 4 and 6, hexokinase and glucose (enzyme system II) were left out as well during the initial 5 minute incubation at 37°C. After incubation, they were heated to 52°C. for 6 minutes, then rapidly cooled. This destroyed enzymatic activity in the crude enzyme inoculum (enzyme system I). System II was then added and after a further incubation of 5 minutes, System III was added and the reaction was followed on a Bausch and Lomb model 505 recording spectrophotometer. The effect of heating System I was noted by running the same procedure without heating tubes 3 and 5.

(e) Xanthosine-5'-Monophosphate Aminase Activity

The activity of this system was determined by employing

the assay of Abrams and Bentley (1959).

The reaction mixture consisted of:

1.0M Tris buffer pH 8.5

0.05M ATP

0.4M $MgCl_2$

0.1M XMP

2.0M NH_4Cl

1.0M glutamic acid

0.1M salicylic acid

disrupted cell inoculum

The reaction mixtures were set up according to the following protocol:

Materials	(Volume in ml.)				
Tube	1	2	3	4	5
Tris	0.04	0.04	0.04	0.04	0.04
$MgCl_2$	0.01	0.01	0.01	0.01	0.01
XMP	0.05	0.05	0.05	0.05	0.00
ATP	0.02	0.02	0.02	0.02	0.02
NH_4Cl	0.04	0.04	0.00	0.00	0.00
glutamic acid	0.02	0.00	0.02	0.02	0.00
salicylic acid	0.02	0.00	0.00	0.02	0.00
HOH	0.00	0.04	0.06	0.04	0.13
inoculum	0.05	0.05	0.05	0.05	0.05

The final volume in all cases was 0.25 ml. The inoculum was added last.

The reaction mixtures were incubated at 37°C. for 30 minutes, then stopped by the addition of 2.75 ml. of 3.5% w/v perchloric acid. The tubes were then centrifuged to remove precipitated protein. The OD's of the deproteinized samples were measured at 290 mμ in a Beckman model DU spectrophotometer to detect the presence of GMP.

Experimental and Results

1. Growth Response to Various Nutrients

In the determination of response to NH_4^+, 26 fifty ml. Erlenmeyer flasks containing 20 ml. of synthetic medium were inoculated at zero time and left to incubate at room temperature with continuous agitation. At the time intervals designated in Table 1, duplicate flasks were removed, and their turbidities measured and averaged. Cells were removed by centrifugation, and the NH_4^+ content of three 1 ml. samples from each supernatant were measured and averaged. The results are seen in Table 1 and Figure 1.

The cultures measured at zero time showed the initial NH_4^+ concentration to be 11.5 μM/ml. The initial population of cells in each culture was 0.061 mg. dry weight of cells per ml.

In the early stages of growth of the cultures, a significant loss (32.2%) of the NH_4^+ in the medium indicated the ion to be essential in the metabolism of the organism. NH_4^+ was taken up at a steady rate during this early growth period of the culture. As the culture entered the logarithmic growth phase, active deaminase activity resulted in an evolution of NH_4^+ as evidenced by an increase of extracellular NH_4^+ in the medium.

Nine ml. of synthetic medium were dispensed in 50 ml. flasks for the determination of growth response to various energy sources. Sterile solutions of various energy sources were added to 9 ml. portions of synthetic medium to form a final concentration of 0.55 μM/ml. The flasks were inoculated

Table 1

NH_4^+ Uptake by Micrococcus sodonensis and Its Effect on Growth Response

Age of Culture (hr.)	Growth Response			NH_4^+ Uptake		
	OD (600 mμ)	Dilution Factor	Dry Wt. mg./ml.	OD (400 mμ)	Dilution Factor	μM NH_4^+/ml.
0	0.209	1	0.061	0.505	10	11.5
2	0.247	1	0.076	0.453	10	10.3
4	0.350	1	0.118	0.430	10	9.7
6	0.547	1	0.198	0.430	10	9.7
8	0.465	2	0.328	0.415	10	9.5
10	0.795	2	0.610	0.403	10	9.1
12	0.875	2	0.668	0.346	10	7.8
20	0.460	10	1.63	0.500	10	11.5
24	0.640	10	2.38	0.570	10	13.1
30	0.475	20	3.38	0.680	10	15.8
37	0.463	20	3.28	0.750	10	17.5
45	0.450	20	3.20	0.700	10	16.3
48	0.455	20	3.22	0.680	10	15.8

Figure 1

NH_4^+ Uptake by Micrococcus sodonensis

and Its Effect on Growth Response

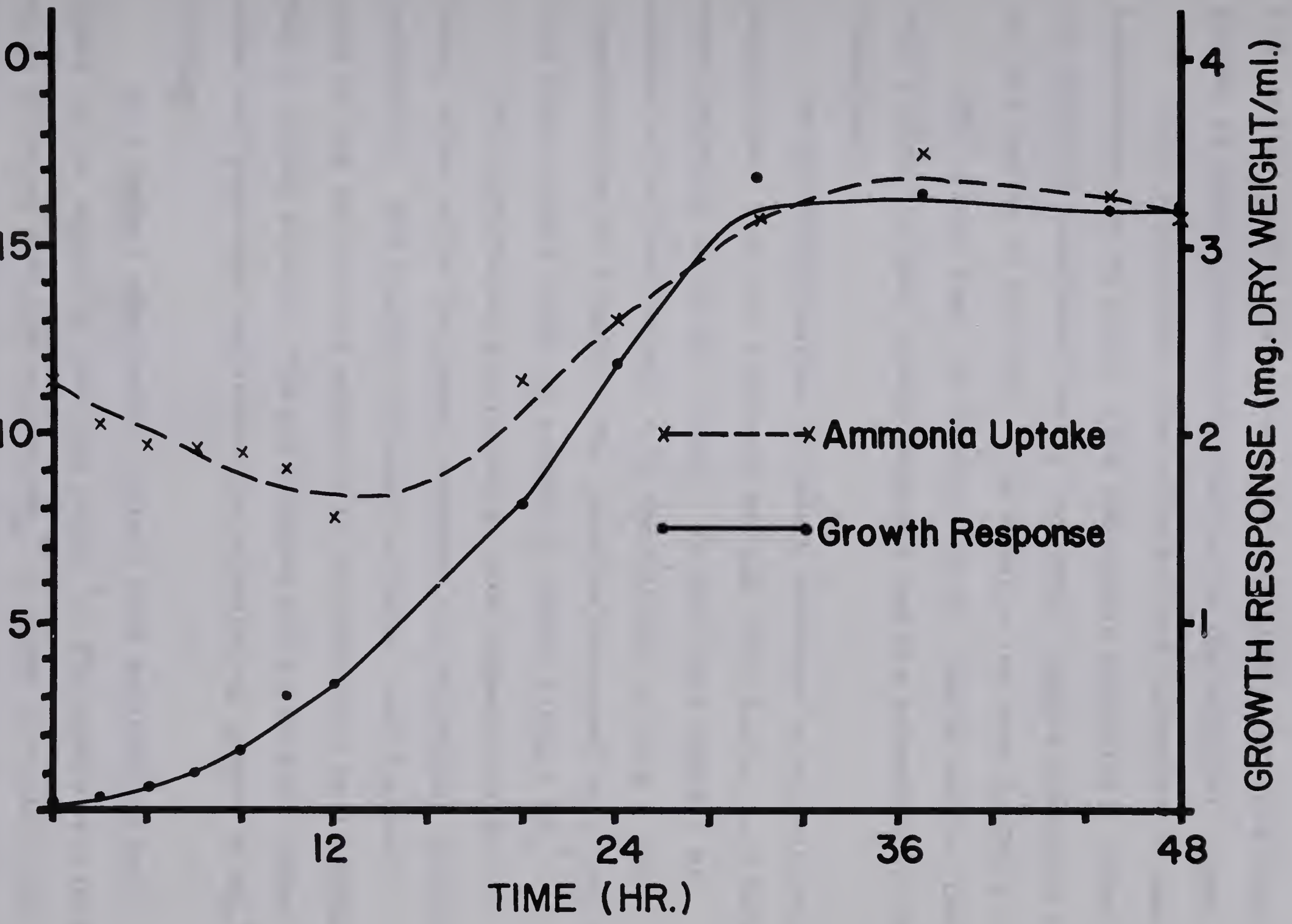

20
15
10
5
μM NH3
4
3
2
1
GROWTH RESPONSE (mg. DRY WEIGHT/ml.)
Ammonia Uptake
Growth Response
12
24
36
48
TIME (HR.)

FIGURE 1

with 0.1 ml. of cells from cells of a 24 hour culture grown in T.S. broth, washed three times in distilled water, and resuspended in sterile distilled water to 5x the original volume.

After inoculation, the cultures were incubated at room temperature for 48 hours, with continuous agitation. Growth was measured turbidimetrically and these figures converted to mg. dry weight of cells/ml. The results are seen in Table 2.

NH_4^+ alone does not function as an energy source. Lactic acid yields the best growth response and was subsequently used throughout.

In the determination of growth response to nucleic acid derivatives, 9 ml. of synthetic medium, with lactic acid added, were dispensed in 50 ml. flasks. To each were added 1 ml. amounts of various nucleic acid derivatives to form a final concentration of 1 μM/ml. The inoculum consisted of 1 drop of a 24 hour culture grown in synthetic medium without NH_4^+.

The cultures were agitated at room temperature for 40 hours, after which the growth response was determined turbidometrically. The results are seen in Table 3 and Figure 2. Many of the derivatives appeared to be inhibitory while thymine had no effect at all. The pyrimidines and pyrimidine nucleosides and guanosine duplicated the stimulatory effect of NH_4^+ closely.

In a second experiment, nucleic acid derivatives were added to the medium in addition to NH_4^+. The growth obtained after 48 hours with and without NH_4^+ is shown in Table 4 and Figure 3. The higher growth response was due to the greater incubation time, and the incubation temperature which was

Table 2

Growth Response of Micrococcus sodonensis to Various Energy Sources

Growth Medium	O.D. (600 mμ)	Dry Wt. (mg./ml.)
Basal + NH_4^+	0.300	0.098
Basal + NH_4^+ + lactic acid	1.380	0.518
Basal + NH_4^+ + xylose	0.241	0.074
Basal + NH_4^+ + arabinose	0.319	0.105
Basal + NH_4^+ + sucrose	0.630	0.232
Basal + NH_4^+ + glycerol	0.284	0.091
Basal + NH_4^+ + galactose	0.441	0.155
Basal + NH_4^+ + glucose	0.535	0.194
Basal + NH_4^+ + fructose	0.253	0.079
Basal + NH_4^+ + ethanol	0.635	0.234
Basal + NH_4^+ + maltose	0.693	0.260
Basal + NH_4^+ + mannitol	0.236	0.062
Basal + NH_4^+ + raffinose	0.266	0.084
Basal + NH_4^+ + inulin	0.433	0.152
Basal + NH_4^+ + sorbitol	0.296	0.096
Basal + NH_4^+ + dextrin	0.443	0.156

Table 3

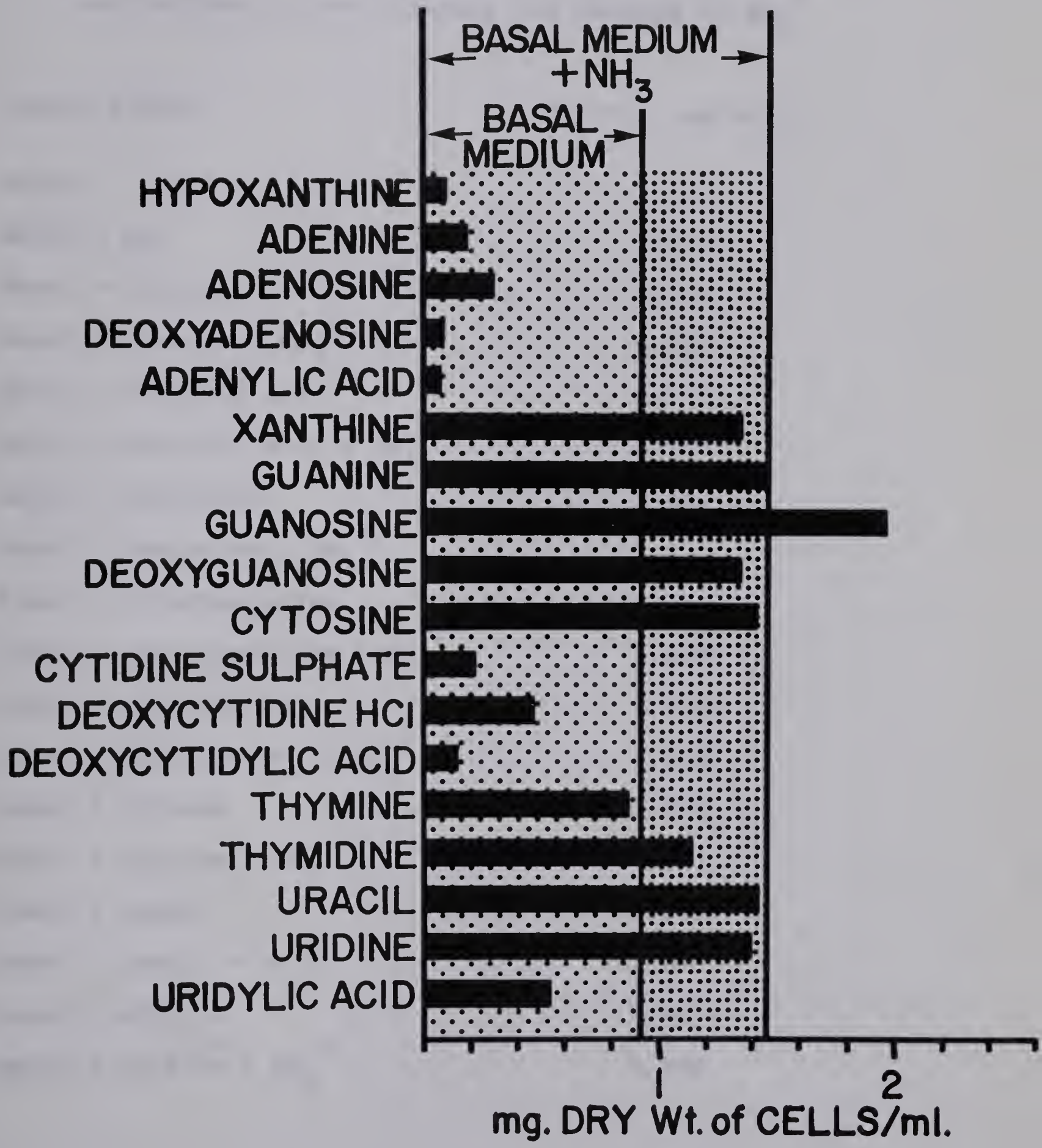
Growth Response of M. sodonensis to Various Nucleic Acid Derivatives

Growth Medium	Dry Wt. (mg./ml.)
Basal	0.950
Basal + NH_4^+	1.460
Basal + hypoxanthine	0.094
Basal + xanthine	1.375
Basal + adenine	0.192
Basal + guanine	1.430
Basal + cytosine	1.410
Basal + thymine	0.875
Basal + uracil	1.410
Basal + adenosine	0.295
Basal + cytidine sulphate	0.208
Basal + guanosine	1.960
Basal + uridine	1.390
Basal + thymidine	1.140
Basal + deoxyadenosine	0.060
Basal + deoxyguanosine	1.350
Basal + deoxycytidine HCl	0.465
Basal + adenylic acid	0.061
Basal + uridylic acid	0.540
Basal + deoxycytidylic acid	0.142

Figure 2

Growth Response of M. sodonensis to Various Nucleic Acid Derivatives

FIGURE 2


BASAL MEDIUM + NH_3
BASAL MEDIUM
HYPOXANTHINE
ADENINE
ADENOSINE
DEOXYADENOSINE
ADENYLIC ACID
XANTHINE
GUANINE
GUANOSINE
DEOXYGUANOSINE
CYTOSINE
CYTIDINE SULPHATE
DEOXYCYTIDINE HCl
DEOXYCYTIDYLIC ACID
THYMINE
THYMIDINE
URACIL
URIDINE
URIDYLIC ACID
1
2
mg. DRY Wt. of CELLS/ml.

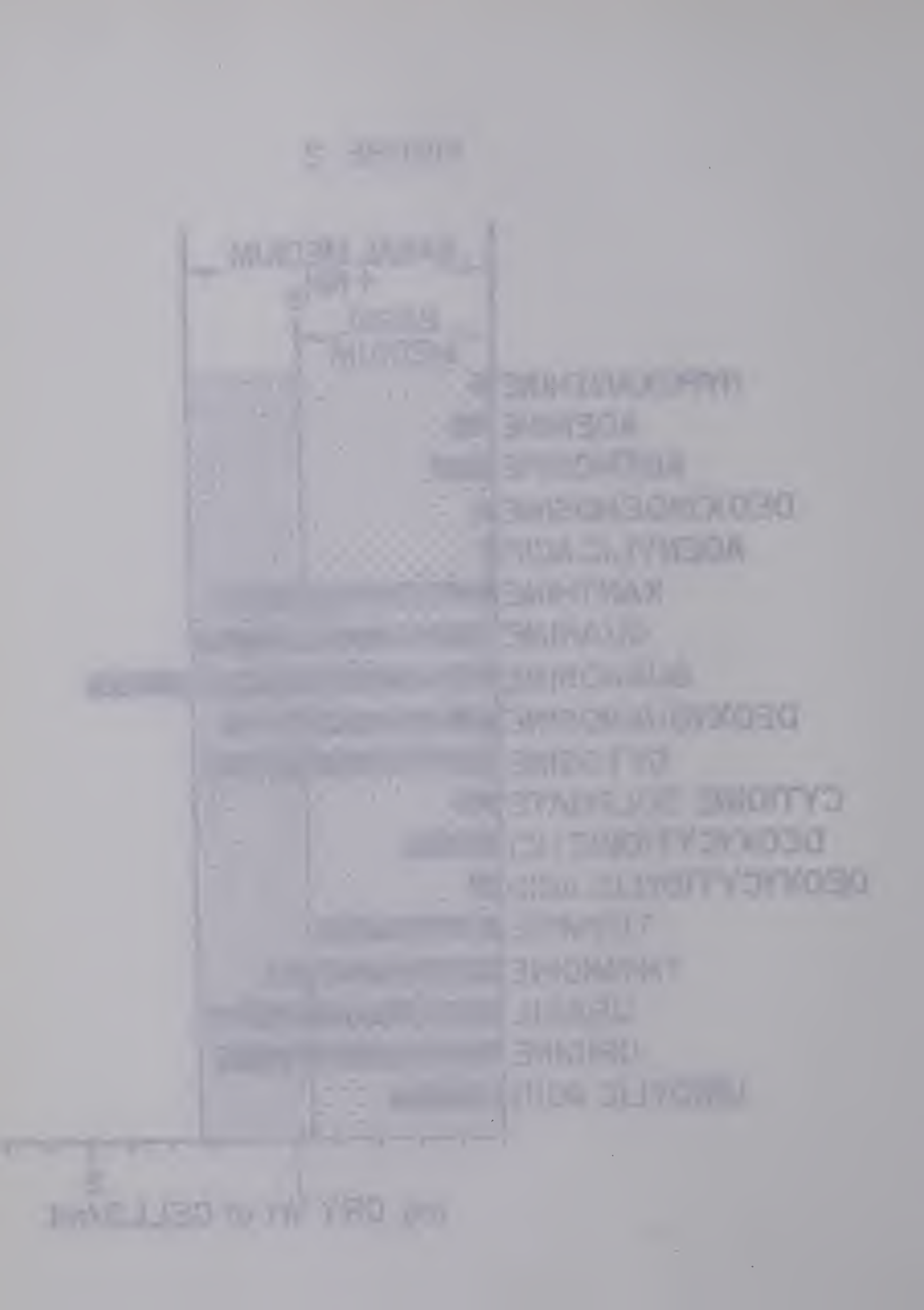


Table 4

Growth Response of M. sodonensis to Various Nucleic Acid Derivatives in the Presence and Absence of NH_4^+

Growth Medium	Dry Wt. (mg./ml.)
Basal	2.310
Basal + NH_4^+	2.860
Basal + adenine	0.390
Basal + adenine + NH_4^+	0.980
Basal + adenylic acid	0.126
Basal + adenylic acid + NH_4^+	0.110
Basal + guanosine	3.600
Basal + guanosine + NH_4^+	3.600
Basal + deoxyguanosine	3.360
Basal + deoxyguanosine + NH_4^+	3.360
Basal + cytidine SO_4	1.480
Basal + cytidine SO_4 + NH_4^+	3.500
Basal + thymine	2.280
Basal + thymine + NH_4^+	3.220
Basal + uracil	2.860
Basal + uracil + NH_4^+	3.260
Basal + uridine	2.410
Basal + uridine + NH_4^+	3.450

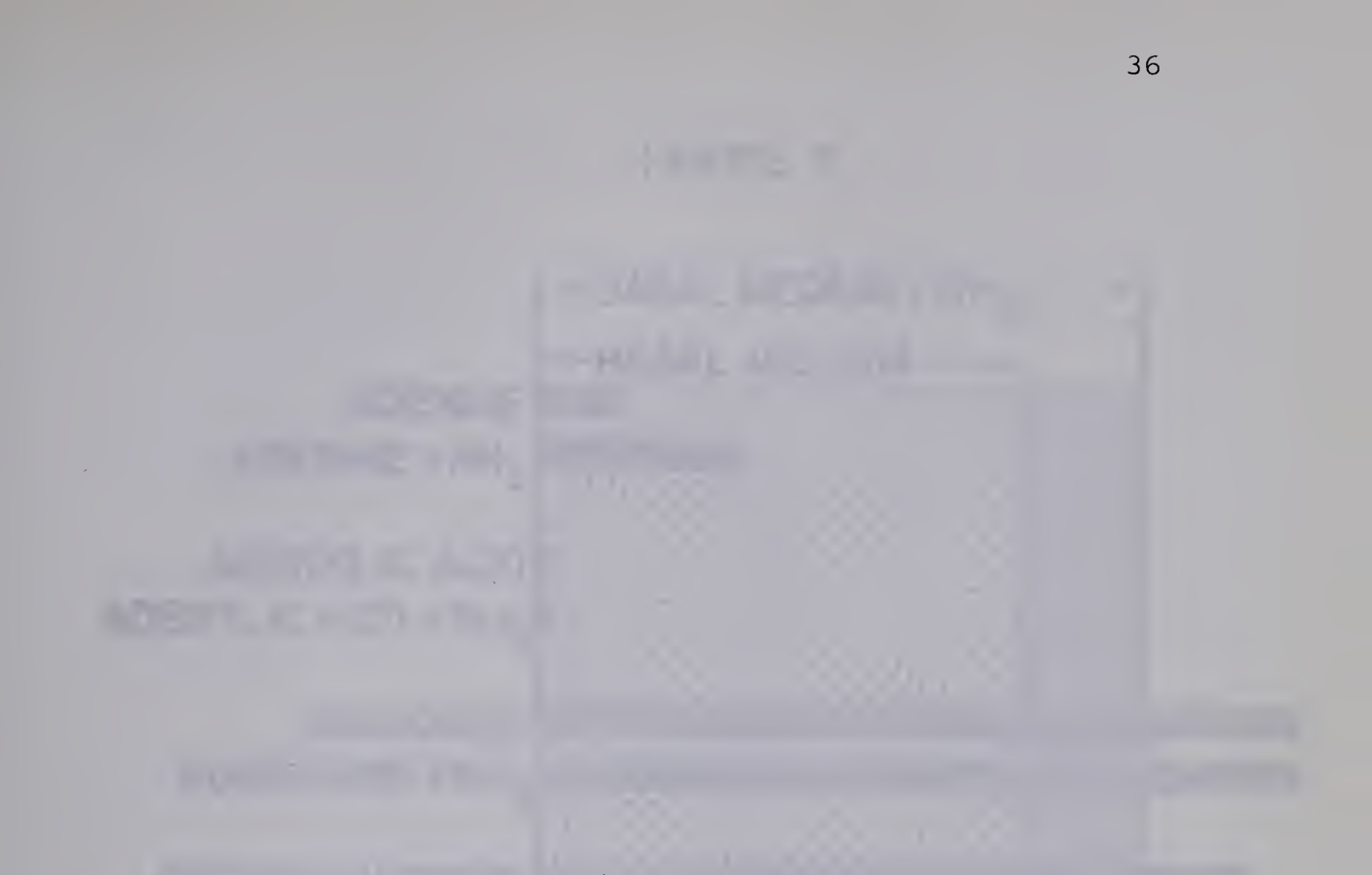
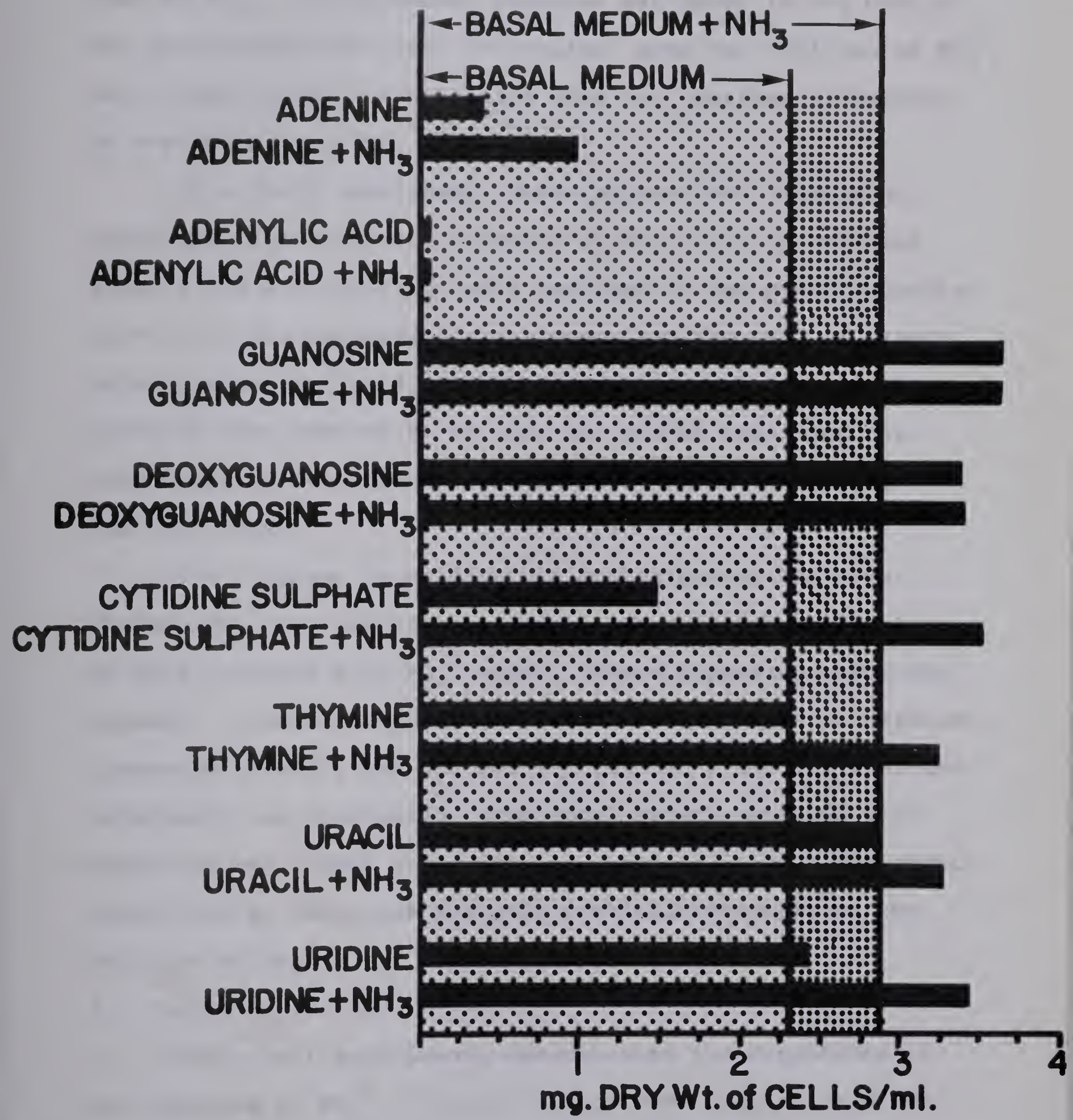


Figure 3

Growth Response of M. sodonensis to Various Nucleic Acid Derivatives in the Presence and Absence of NH_4^+

FIGURE 3


BASAL MEDIUM + NH3
BASAL MEDIUM
ADENINE
ADENINE + NH3
ADENYLIC ACID
ADENYLIC ACID + NH3
GUANOSINE
GUANOSINE + NH3
DEOXYGUANOSINE
DEOXYGUANOSINE + NH3
CYTIDINE SULPHATE
CYTIDINE SULPHATE + NH3
THYMINE
THYMINE + NH3
URACIL
URACIL + NH3
URIDINE
URIDINE + NH3
1
2
3
4
mg. DRY Wt. of CELLS/ml.

approximately 4°C. higher.

The inhibitory effect of adenine was only slightly overcome by NH_4^+. An increased response was noted in the case of the pyrimidines and their nucleosides upon the addition of NH_4^+. Cells grown in NH_4^+ + guanosine were not further stimulated by the addition of NH_4^+.

In a third experiment, three nucleosides (adenosine, guanosine, and uridine), selected on the basis of previous results and availability, were employed at the same concentration as in the preceeding experiments and the rate of growth with time was followed. (Table 5 and Figure 4). Duplicate cultures were removed at 12, 24, 36, and 60 hours and the turbidimetric measurements of growth measured and averaged for each time.

The response to uridine was approximately the same whether NH_4^+ was present or absent. This response was close to that obtained with NH_4^+ alone. Where guanosine alone was present, a slower response was noted, but the rate of response increased steadily and had not yet reached a maximum when the experiment was terminated. The total growth obtained with guanosine was higher than with any other additive. The growth inhibition by adenosine was only partially overcome by the addition of NH_4^+.

2. Enzyme Activities

Whole cell experiments demonstrated the dependence of the organism on NH_4^+. The ability of some nucleic acid derivatives to mimic the stimulatory effect of NH_4^+ implicated several possible enzyme systems which use NH_4^+ as a substrate.

Table 5

Rate of Growth of M. sodonensis in the Presence of Adenosine, Guanosine, and Uridine With and Without the Addition of NH_4^+

Growth Medium	Growth Response (dry wt. mg./ml.)			
	12 hr.	24 hr.	36 hr.	60 hr.
Basal + NH_4^+	0.204	1.840	3.240	3.680
Basal alone	0.150	0.900	2.400	3.040
Guanosine	0.140	1.180	2.220	4.160
Guanosine + NH_4^+	0.163	1.300	3.240	3.720
Adenosine	0.102	0.140	0.240	0.460
Adenosine + NH_4^+	0.094	0.210	0.490	2.460
Uridine	0.184	1.350	3.040	3.960
Uridine + NH_4^+	0.222	2.220	3.240	3.940

Figure 4

Rate of Growth of M. sodonensis in the Presence of Adenosine, Guanosine, and Uridine With and Without the Addition of NH_4^+

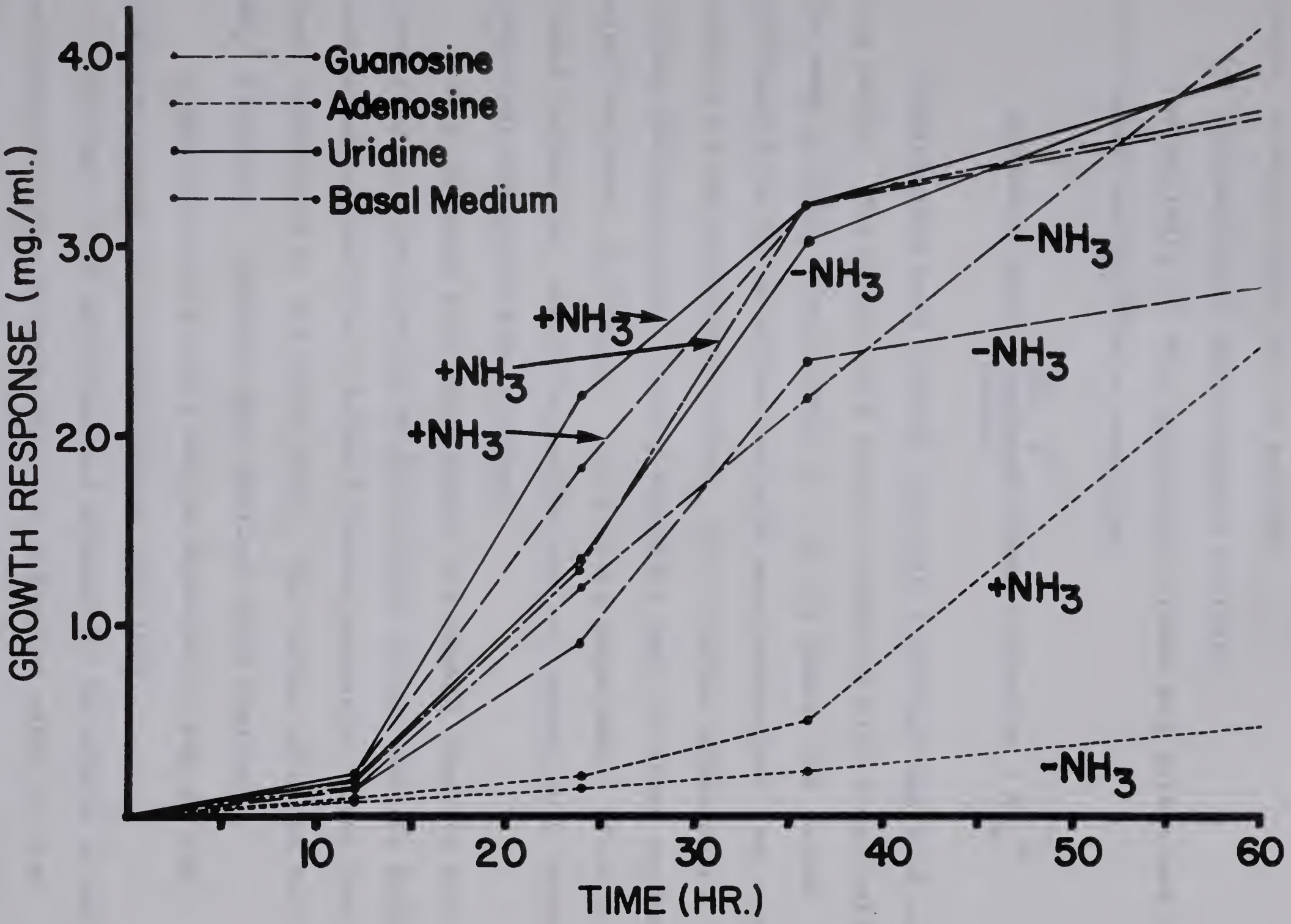
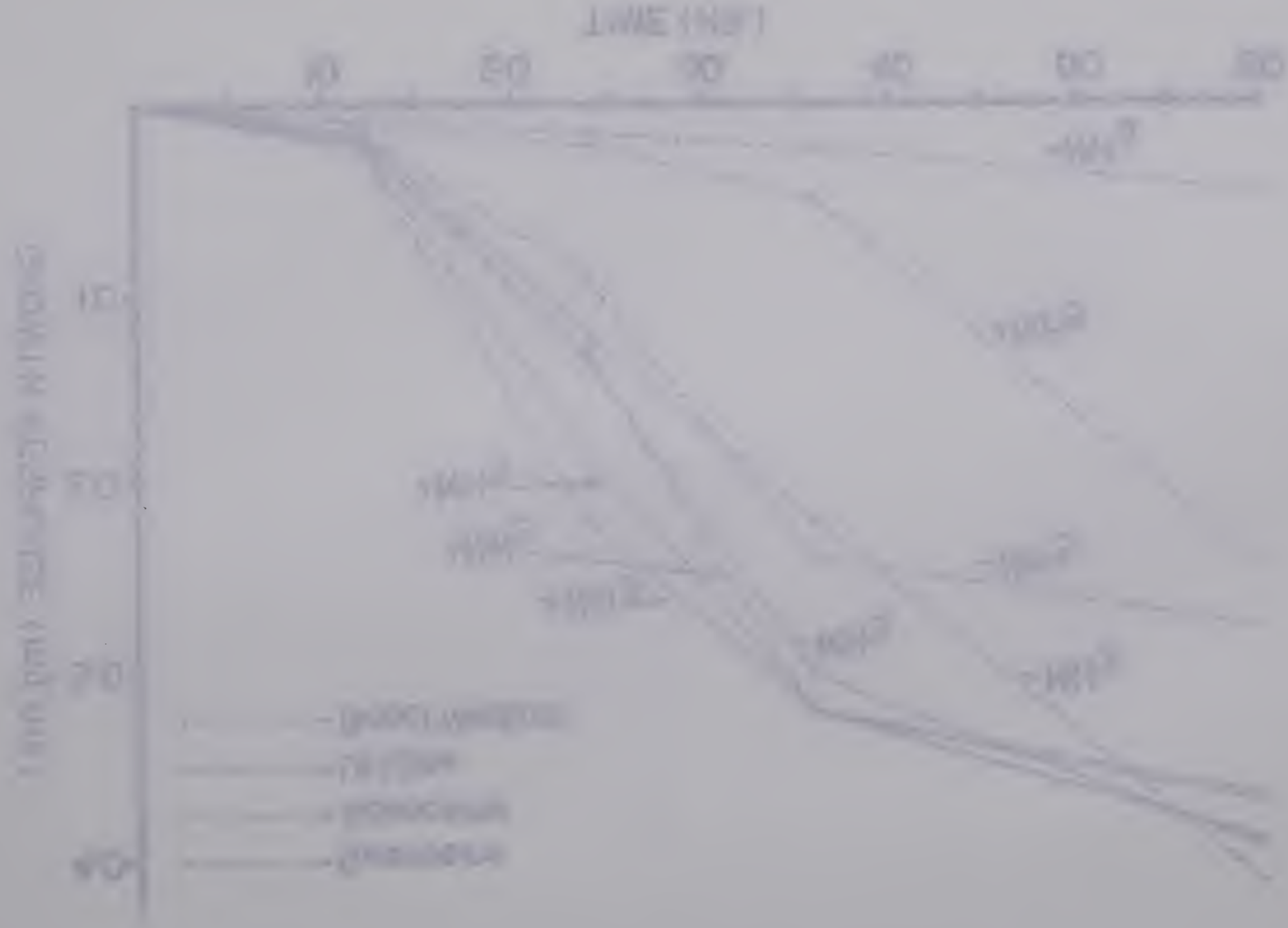

Guanosine
Adenosine
Uridine
Basal Medium
GROWTH RESPONSE (mg./ml.)
4.0
3.0
2.0
1.0
+NH3
+NH3
+NH3
-NH3
-NH3
-NH3
+NH3
-NH3
10
20
30
40
50
60
TIME (HR.)

FIGURE 4



The synthesis of glutamic acid was another route of NH_4^+ incorporation which could not be excluded.

(a) Glutamic Acid Dehydrogenase Activity

The activity of this reversible system was determined by the Conway microdiffusion technique.

$$\text{glutamic acid} \underset{\text{deHase}}{\overset{\text{glu } \bar{a}}{\rightleftharpoons}} \alpha\text{-keto-glutarate} + NH_4^+$$

Experiments were carried out with fresh and depleted resting cells. The result of 2 hours incubation with fresh cells is seen in Table 6 and Figure 5. In all cases, the values obtained from the N-saline controls were subtracted from those obtained from inoculated plates. In a similar experiment, the values obtained after 1 hour incubation were in close agreement with the curve in Figure 5. In a third experiment, after 4 hours incubation, total demination was not appreciably increased over that obtained after 2 hours.

In a depleted cell experiment, the washed inoculum was incubated in distilled water for 2 hours before use, to deplete endogenous metabolites. After 4 hours incubation under the same conditions as the fresh cells, the values obtained for NH_4^+ evolution, although much smaller, were similar.

α-keto-glutarate could not be detected in any of the cultures using the electrophoretic technique.

Glutamic acid dehydrogenase appears to be active in the metabolism of the organism and was discerned early in the incubation period. In the absence of an energy source, the organism was seen to deaminate glutamic acid to obtain α-keto-

Table 6

Deaminase Activity of Fresh Resting Cells of M. sodonensis

Reaction Mixture	μM/ml. of NH_4^+ evolved after 2 hours
cells + buffer	0.20
cells + NH_4^+	0.15
cells + glutamic acid	0.40
cells + glutamic acid + NH_4^+	0.40
cells + lactic acid	0.00
cells + lactic acid + NH_4^+	0.00
cells + lactic acid + glutamic acid	0.00
cells + lactic acid + glutamic acid + NH_4^+	0.00

Figure 5

Deaminase Activity of Fresh Resting Cells of M. sodonensis

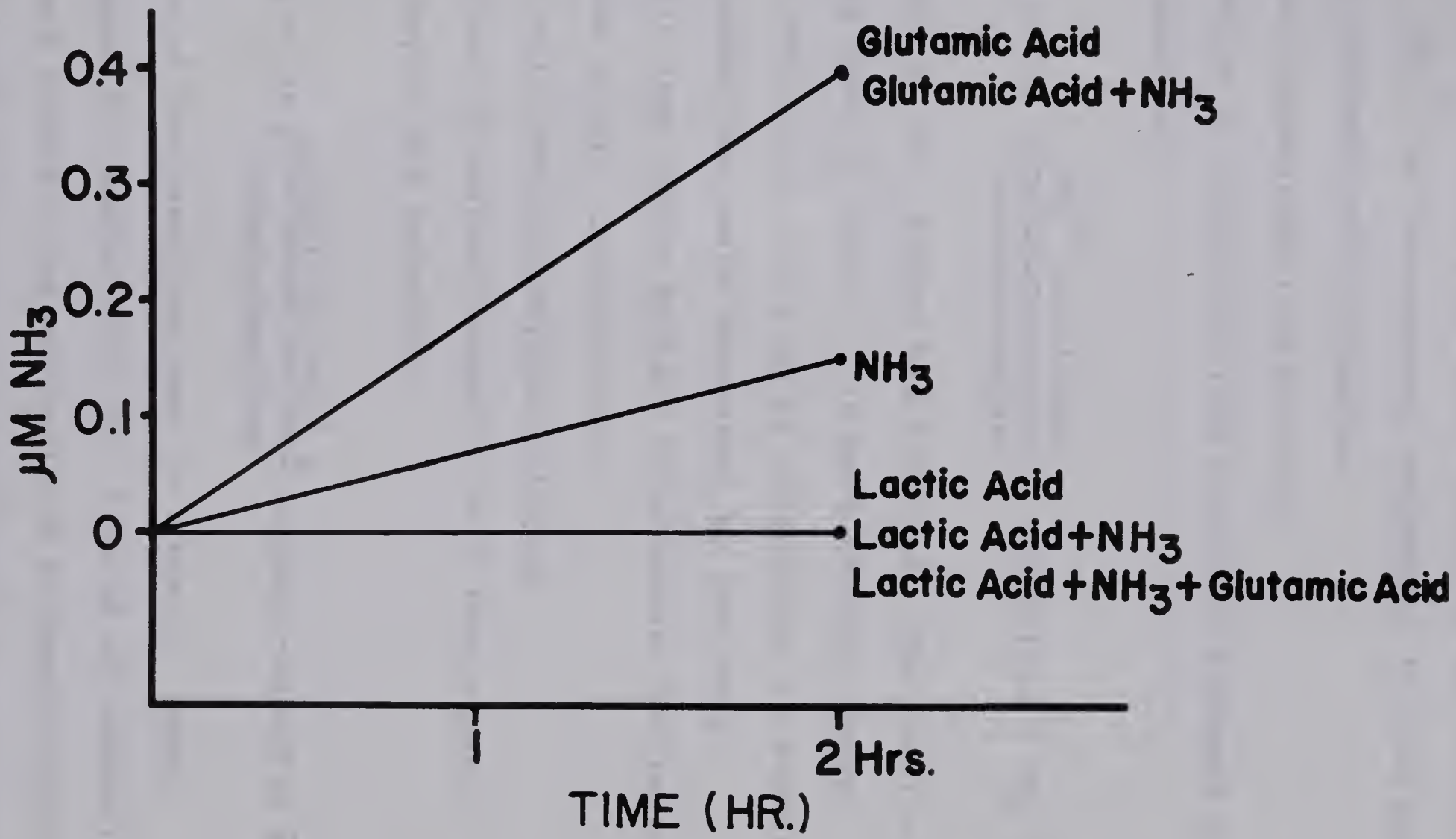
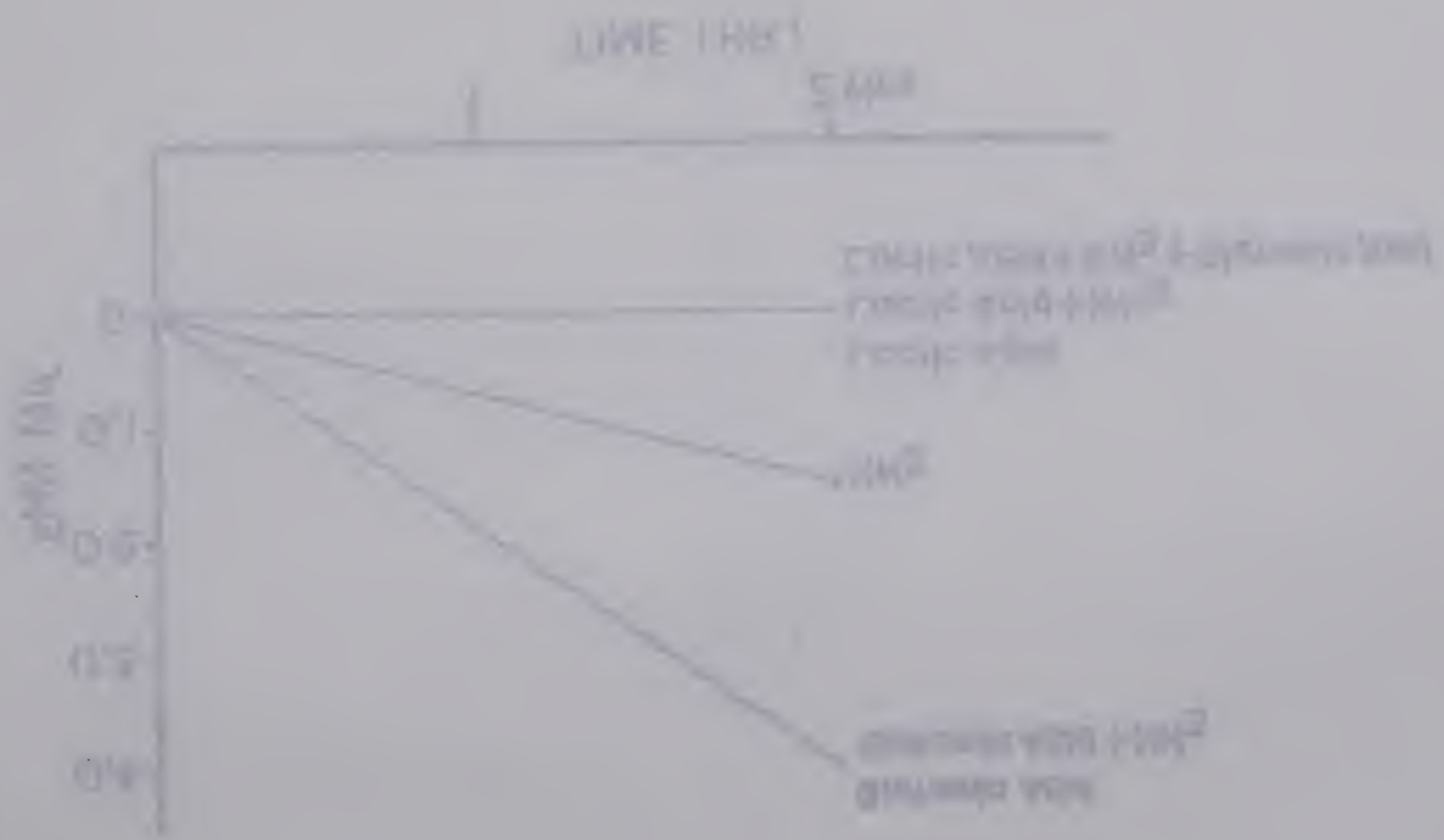

0.4
0.3
0.2
0.1
0
μM NH3
Glutamic Acid
Glutamic Acid + NH3
NH3
Lactic Acid
Lactic Acid + NH3
Lactic Acid + NH3 + Glutamic Acid
1
2 Hrs.
TIME (HR.)

FIGURE 5



glutarate. That α-keto-glutarate produced was used by the organism was evidenced by the absence of this energy source in the medium. NH_4^+, an unnecessary by-product for the resting cells, was released into the medium.

(b) Ornithine Transcarbamylase Activity

The production of citrulline from ornithine proceeds by the following reaction:

$$\text{ornithine} + \text{CAP} \xrightleftharpoons[\text{transcarbamylase}]{Mg^{++}\ \text{ornithine}} \text{citrulline} + PO_4^{-3}$$

The presence of this enzyme was detected. The results of the assay are seen in Table 7. In each reaction vessel, under complete conditions, 0.072 μM of citrulline, in excess of endogenous production, were determined by the assay.

This initial step before protein biosynthesis indirectly indicated a route of NH_4^+ incorporation.

(c) Aspartic Acid Transcarbamylase Activity

The production of carbamyl-aspartic acid from aspartic acid occurs by way of the reaction:

$$\text{L-aspartic acid} + \text{CAP} \xrightleftharpoons[\text{transcarbamylase}]{\text{aspartic acid}} \text{carbamyl-aspartic a} + PO_4^{-3}$$

The results of the assay are seen in Table 8. Under complete conditions for enzyme activity, 0.91 μM of carbamyl-aspartic acid were formed. The synthesis of carbamyl-aspartic acid ultimately leads to pyrimidine biosynthesis and involves the incorporation of NH_4^+.

Table 7

Demonstration of Ornithine Transcarbamylase Activity in Cell-Free Preparations of Micrococcus sodonensis

Reaction Tube	OD (490 mμ)	citrulline (μM/ml.)
1. complete medium + inoculum	0.11 (av)	0.12
2. complete medium minus inoculum	0.03	0.034
3. medium minus ornithine + inoculum	0.02	0.022
4. medium minus CAP + inoculum	0.02	0.022
5. inoculum + buffer only	0.045	0.048

Table 8

Demonstration of Aspartic Acid Transcarbamylase Activity in Cell-Free Preparations of *M. sodonensis*

Reaction Mixture	OD (590 mμ)	carbamyl-aspartic acid (μM/ml.)
1. complete medium + inoculum	0.300 (av)	1.67
2. complete medium minus inoculum	0.130	0.73
3. medium minus CAP + inoculum	0.115	0.65
4. medium minus aspartic acid + inoculum	0.128	0.72
5. inoculum + buffer	0.1375	0.76

(d) Carbamyl Phosphate Synthetase Activity

The production of carbamyl phosphate (CAP) proceeds by the following reaction:

$$NH_4^+ + CO_3^= + ATP \xrightleftharpoons[\text{CAP synthetase}]{Mg^{++}} CAP + ADP$$

The experiments previously described clearly demonstrated that the organism employed CAP in the synthesis of other intermediates. The synthesis of CAP, involving the incorporation of NH_4^+, remained to be determined.

Activity of the enzyme could not be discerned with disrupted cell preparations incubated with NH_4^+, $CO_3^=$, HCO_3^- and ATP, employing the assays for citrulline and carbamyl-aspartic acid. The method of Fiske and SubbaRow was tried and still no direct production of CAP was demonstrable. Attempts to demonstrate the de novo synthesis of CAP from elemental components were unsuccessful using any of the three assay techniques.

The activity of CAP synthetase was observed by reversing the equilibrium of the enzyme system and assaying for the formation of ATP by disrupted cells as shown in the following scheme:

System I $\quad CAP + ADP \xrightarrow[\text{CAP synthetase}]{Mg^{++}} ATP + CO_2 + NH_4^+$

System II $\quad glucose + ATP \xrightarrow{\text{hexokinase}} G\text{-}6\text{-}P + ADP$

System III $\quad G\text{-}6\text{-}P \text{ deHase}$, $NADP^+$ → 6-phosphogluconolactone + $NADPH \cdot H^+$

The assay for ATP employed the hexokinase-glucose-6-phosphate dehydrogenase system and was followed continuously on a recording spectrophotometer at 340 mμ to determine the formation of $NADPH \cdot H^+$.

The results are seen in Figure 6. Tube 1 was a control containing hexokinase and glucose (System I) and G-6-P deHase and $NADP^+$ (System II). The addition of ATP resulted in the formation of $NADPH \cdot H^+$, as was seen in Tube 2. Tube 3, a control, contained the inoculum and ADP (System I), plus Systems II and III. The addition of CAP resulted in the formation of $NADPH \cdot H^+$ as was seen in Tube 5. Tube 4 was a control identical to Tube 3, but one more step was included in the procedure. System I was incubated separately, then inactivated before the addition of Systems II and III. Tubes 5 and 6 were the same except for a similar heating step in Tube 6. Systems II and III were acting on the same substrate, ATP, but System I was inactivated in Tube 6.

It was noted that $NADPH \cdot H^+$ was produced in both tubes

Figure 6

Carbamyl Phosphate Synthetase Activity in a Disrupted Cell Preparation of M. sodonensis

Legend

Tube 1 Systems II and III

Tube 2 Systems II and III and ATP

Tube 3 Systems I, II and III

Tube 4 Inactivated System I and Systems II and III

Tube 5 Systems I, II, III and CAP

Tube 6 Inactivated System I and Systems II, III and CAP

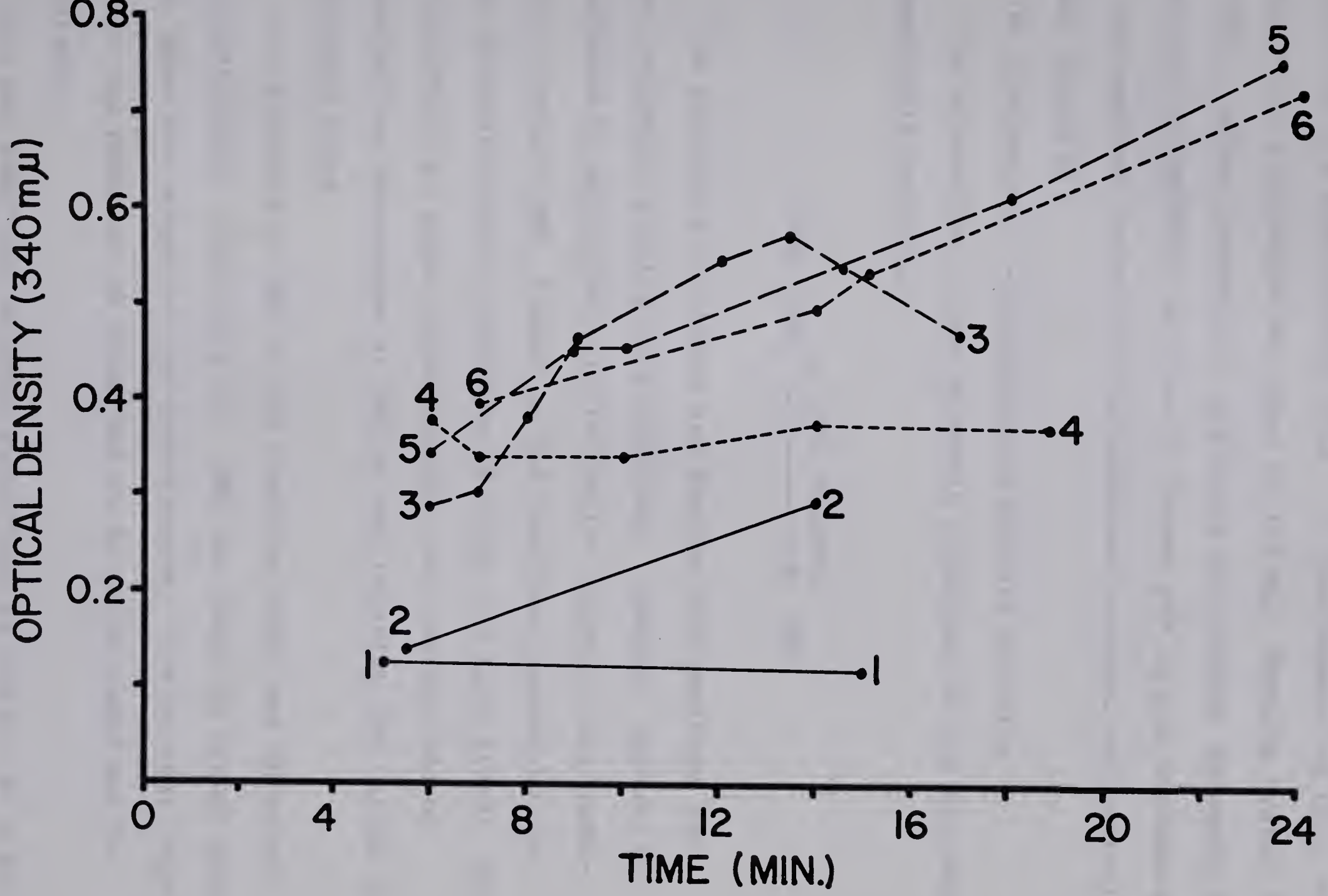
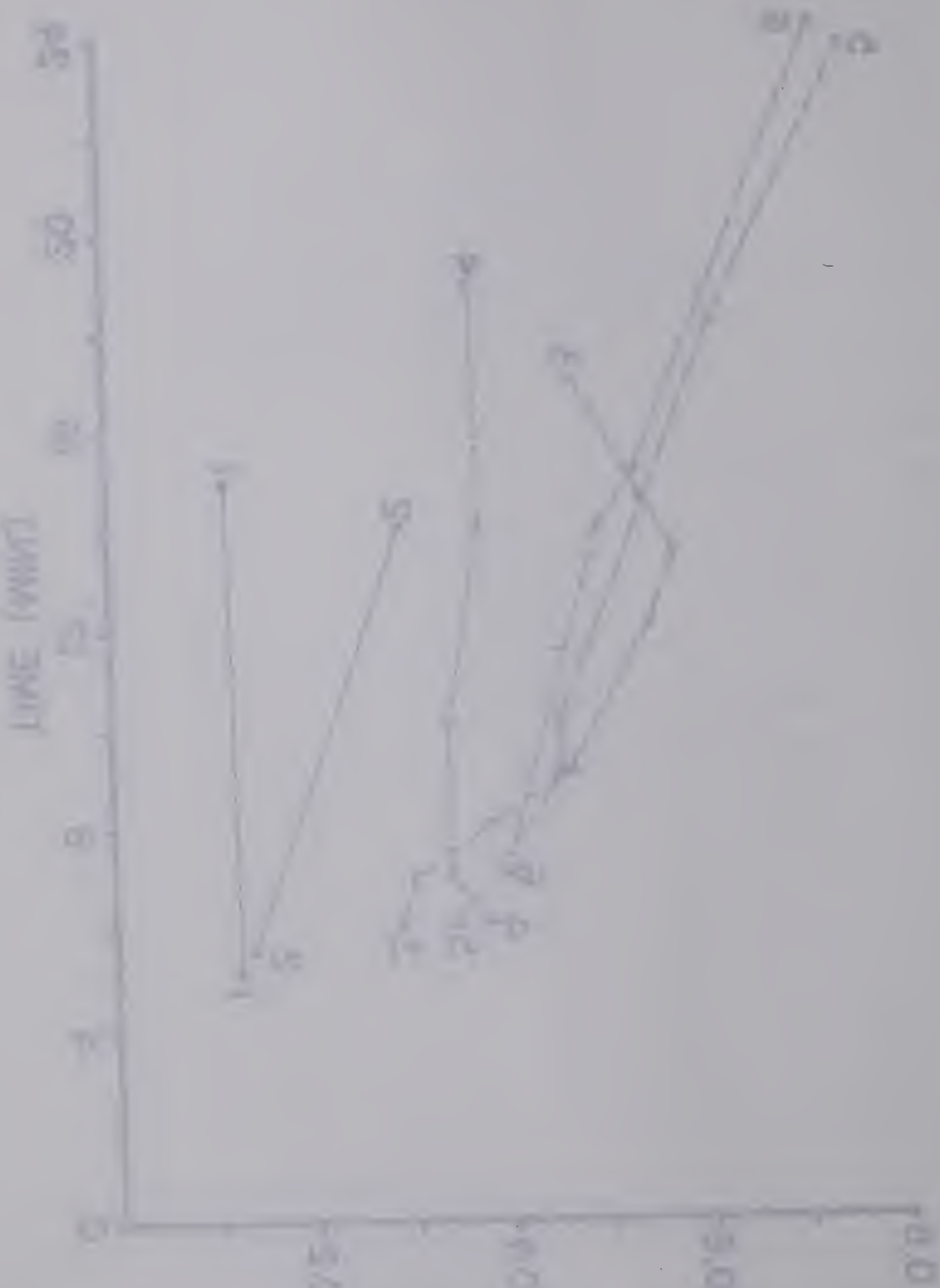

OPTICAL DENSITY (340mμ)
0.8
0.6
0.4
0.2
0
4
8
12
16
20
24
TIME (MIN.)
1
2
3
4
5
6

FIGURE 6



5 and 6 at the same uniform rate. In the absence of CAP, no increase in absorbance was seen at 340 mμ. Heating System I to 52°C., after incubation, prevented any further endogenous evolution of $NADPH \cdot H^+$ or loss of formed ATP as was evidenced by the smoother curves of the preheated reactant and control tubes (6 and 4).

(e) Xanthosine-5'-Monophosphate Aminase Activity

The production of GMP proceeds according to the following irreversible reaction:

$$XMP + NH_4^+ \xrightarrow{\text{XMP aminase}} GMP$$

The procedure of Abrams and Bentley was employed and some favourable results were obtained. However, because of absorbance by salicylic acid in the UV range, it was necessary to isolate GMP, formed in the reaction mixture,by the paper chromatographic technique of Singh and Lane (1963). The GMP separated by paper chromatography was quantitated by measuring the absorbance at 260 mμ and relating the values to a standard curve.

The presence of GMP in the reaction tubes was detected and quantitated. (See Table 9). GMP was found to travel about 5 cm. from the origin on the chromatogram. The solvent front ran off the paper at 14 hours and an Rf value could not be calculated.

The GMP exhibited maximum absorption at 260 mμ, at a pH of 2, rather than 257 mμ. This was probably due to trace amounts of endogenous CMP which did not separate completely

Table 9

Xanthosine-5'-Monophosphate Aminase Activity
in Disrupted Cell Preparations
of Micrococcus sodonensis

Reaction Plate	OD (260 mμ)	GMP (μM/ml.)
1. NH_4^+ + glutamic acid + AMP + salicyclic acid + inoculum	0.198	0.016
2. NH_4^+ + XMP + inoculum	0.300	0.0245
3. glutamic acid + XMP + inoculum	0.218	0.018
4. glutamic acid + XMP + salicylic acid + inoculum	0.133	0.011
5. inoculum + buffer	0.143	0.012

from GMP on the chromatogram. In the presence of XMP and NH_4^+, 0.0125 μM of GMP were produced by the crude enzyme preparation.

PART II

Isotope Enrichment Studies

A direct determination of the involvement of NH_4^+ in the metabolism of the cell was possible by incorporating $N^{15}H_4Cl$ into the medium and following its appearance, with time, in several fractions of the cell.

Materials and Methods

1. Cellular Extractions

(a) Amino Acid Pool

Forty-five ml. of a 24 hour culture of Micrococcus sodonensis were washed and centrifuged 3 times. The cells were resuspended in 3 ml. of distilled water, put in a boiling water bath for 5 minutes and then centrifuged. The supernatant, containing the amino acid pool, was retained and the cell residue was saved for subsequent extraction of the early nucleotide pool.

(b) Early Nucleotide Pool

The residue was washed twice in cold, distilled water, suspended in 5 ml. of cold 10% TCA and left in an ice bath for 1 hour. At the end of that time, the suspension was centrifuged and the supernatant, containing the early nucleotide pool, was retained.

Nucleotides were separated from organic material in the supernatant by means of activated charcoal (Norite). Approximately 5 gms. (wet weight) of charcoal were added to a 5 ml. supernatant and the suspension was agitated vigorously for 2 hours at room temperature. The suspension was centrifuged at 10,000 rpm for 5 minutes and the supernatant poured off. The charcoal pellet was washed twice in distilled water and resuspended in 10 ml. of 5% pyridine. After vigorous agitation at 37^{o}C. for 4 hours, the suspension was centrifuged and the supernatant, containing the early nucleotide pool, was evaporated to dryness in a flash evaporator. The residue was then resuspended in 3 ml. of distilled water.

(c) Nucleic Acids

A separate, but similar culture was employed to obtain the nucleic acid fraction. A modified procedure of Marmur (1961) was employed for this extraction.

Approximately 50 mg. dry weight of cells were washed and suspended in 20 ml. of 0.035M phosphate buffer (pH 7) containing molar sucrose and 1.0 mg./ml. of lysozyme. The suspension was allowed to digest for 2 hours at 37°C. on a wrist action shaker, after which it was centrifuged at 15,000 rpm for 10 minutes to precipitate the protoplasts formed.

The supernatant was discarded and to the pellet was added 5 ml. of cold distilled water. The suspension was shaken for 5 minutes until viscous, then 5 ml. of cold 2M NaCl and 10 ml. of cold liquified phenol were added. The mixture was agitated for 30 minutes at 6°C., followed by centrifugation at 15,000 rpm for 10 minutes. The top,aqueous layer was removed and extracted 5 times with an equal volume of ether to remove residual phenol.

A chloroform-octanol mixture (8:1) was added twice to remove protein which was seen as a white, denatured layer at the interface of the water-organic solvent mixture.

To the separated aqueous layer was added 1 ml. of a 3M sodium acetate plus 0.001M EDTA solution (pH 7), after which 2 ml. quantities of cold isopropanol were added to the cold solution. The precipitated strands of DNA were centrifuged out of solution. The lower molecular weight RNA was precipitated with cold acetone.

The precipitated nucleic acids were redissolved in M

NaCl and their identity and amount determined by means of orcinol and diphenylamine (DPA) reagents according to the procedure of Schneider (1957). The results obtained were compared to the previously prepared standard curves for DNA and RNA concentration and the colour reactions of orcinol and diphenylamine. (See Appendix - Figures 20, 21, 22, 23).

The standard curve for DNA was prepared by determining the optical densities of various known concentrations of herring sperm DNA (Calbiochem) dissolved in 0.07M phosphate buffer at pH 7. The optical densities were read at 260 mμ on a Beckman model DU spectrophotometer.

A similar procedure was followed to prepare the standard curve for yeast RNA.

The DPA colour reaction was obtained by mixing 1 part DNA solution with 2 parts DPA reagent. The mixture was boiled for 10 minutes and the OD read at 600 mμ on a Bausch and Lomb Spectronic 20 colourimeter.

The orcinol reaction was obtained by mixing equal volumes of RNA solution with orcinol reagent. The mixture was boiled for 20 minutes and the OD at 660 mμ measured on a colourimeter. These colourimetric readings were converted to mg. nucleic acid by comparison with previously prepared standard curves.

2. Mass Spectrometric Analysis of Fractions

Organic nitrogen compounds were converted to gaseous nitrogen for analysis as follows:

Extracted materials were mixed with 5 ml. of cH_2SO_4, 1.0 g. of K_2SO_4, and a selenite boiling chip in a Kjeldahl digestion flask. The organic material was digested by boiling the

mixture for 1 hour until the solution was colourless. The contents of the flask were then transferred quantitatively to a Kjeldahl steam distillation apparatus. To the apparatus was added sufficient 10 N NaOH to make the acid solution basic, and then mild heat was applied to distill off the ammonia.

The released ammonia was collected in an acid trap consisting of 5 ml. of 0.01N H_2SO_4 and 2 drops of methyl red indicator. The acid trap was kept acidic by the addition of more 0.01N HCl as required. Upon completion of ammonia evolution, the acid trap was evaporated to 3 ml. in a flash evaporator and added to the reaction vessel shown in Figure 7.

The NaOBr employed in the oxidation of the NH_4^+ salts was made according to the procedure of Sprinson and Rittenberg (1949). To 150 ml. of 40% w/w NaOH, held at 0°C., were added 50 ml. of bromine - slowly, with vigorous stirring. To this was added another 150 ml. of 40% NaOH. The mixture was stored in a refrigerator and diluted with an equal volume of distilled water before use.

The contents of the reaction flask were frozen to -190°C. by means of liquid air and the atmosphere of the flask was reduced to less than 1×10^{-5} mm pressure with a mercury diffusion pump. The flask was then sealed off, thawed, and an equal volume of hypobromite solution was added. The reaction was allowed to proceed for several minutes, whereupon the reaction vessel was again frozen with liquid air. The nitrogen gas evolved was collected in a breakseal which was immersed in liquid air, and when the gas reached an equilibrium between the two vessels, the breakseal was flamed off.

Figure 7

Diagram of Diffusion Pump, Reaction Vessel, and Collection Apparatus Used in the Evolution of Gaseous Samples

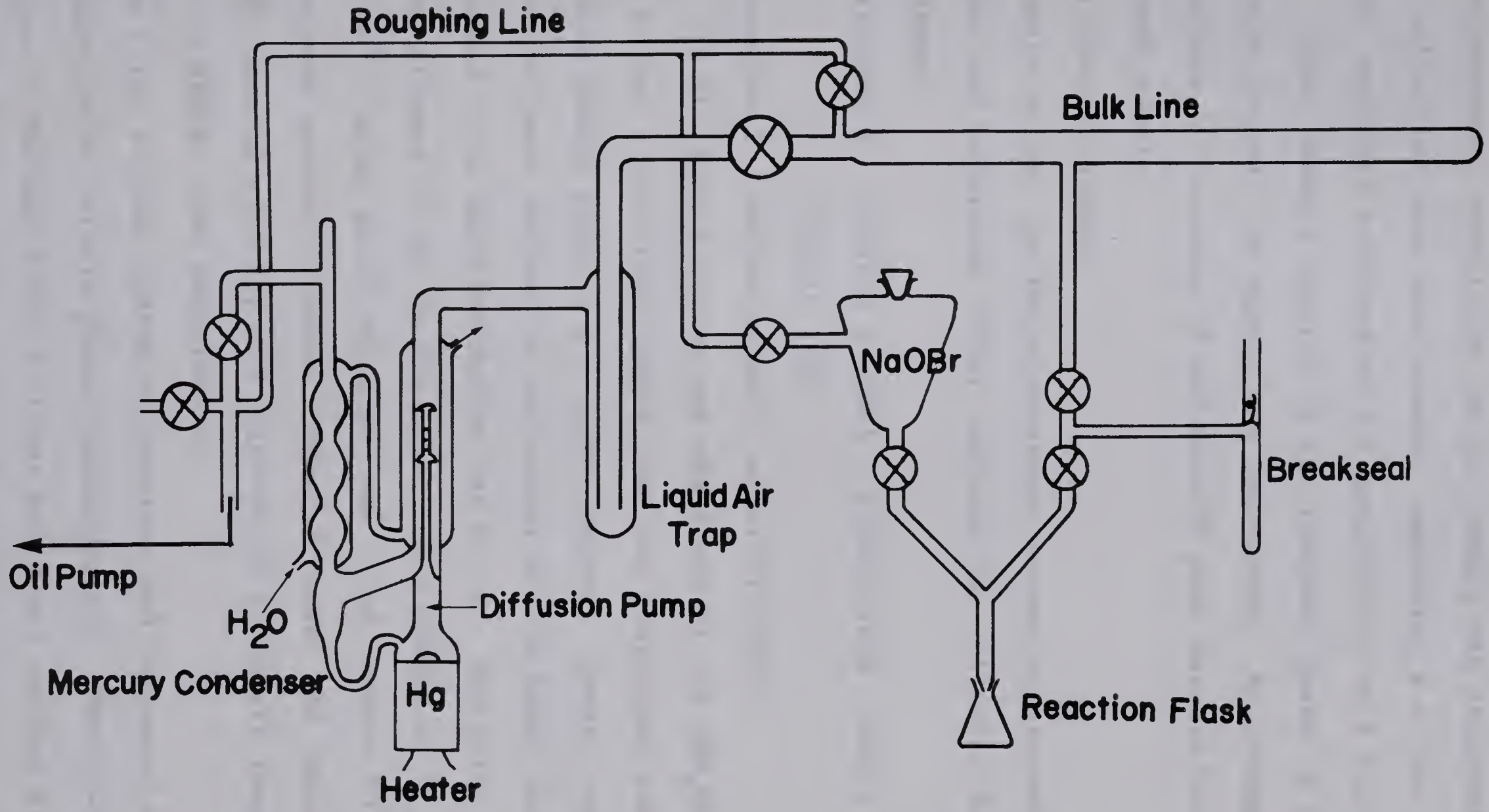

Roughing Line
Bulk Line
NaOBr
Liquid Air
Trap
Breakseal
Oil Pump
H_2O
Diffusion Pump
Mercury Condenser
Hg
Heater
Reaction Flask

FIGURE 7

The breakseal containing the gas sample was attached to a single collection mass spectrometer, employing a 6 inch radius, 60^{o} magnetic analyser with a sensitivity of 1 part in 1000. After several spectra of the residual gases in the machine were traced, the sample was introduced. An average of 20 readings of the mass 28 and mass 29 peak heights were made of each sample.

3. Paper Chromotography

For amino acids, the two-dimensional ascending procedure of Rockland and Underwood (1954) employed the following developing systems:

Direction 1. t-butyl alcohol - formic acid - water (695:10:295)

Direction 2. molten phenol - water (775:215)

To 94 ml. of the second system was added one ml. of cNH_4OH at the time of use. The filter paper used was Schleicher and Schuell Blue Ribbon paper 5 x 5 inches square. Twenty lambda of supernatant were spotted in one corner of the paper which was suspended in an American Museum Jar No. 4. The first system was allowed to run up the paper for a period of 8 hours at 30^{o}C. after which the paper was dried, turned 90^{o}, and the second system initiated. After 6 hours, the paper was dried, sprayed with a 0.25% solution of ninhydrin reagent and left to dry at room temperature.

The first solvent system of Rockland and Underwood was used in descending, single phase chromatography. Twenty-four inch strips of Whatman's No. 1 filter paper was spotted with 100 lambda and left to develop at 30^{o}C. for 24 hours. The

paper was dried at 50°C. and sprayed with ninhydrin.

The paper chromatography procedure of Singh and Lane (1963) was used for the separation of nucleotides. Two hundred lambda of the TCA extract were streaked onto a 24 inch strip of ammonium sulphate-impregnated paper. After running in a descending ethanol-water solvent system for 18 hours, the paper was dried and the UV absorbing areas detected with a UV hand lamp were cut out and eluted in 0.07M phosphate buffer at pH 7.

The procedure of Wyatt (1951) was used to separate nucleic acid bases. Two hundred lambda were spotted onto a 24 inch strip of Whatman's No. 1 filter paper. The solvent system employed was 60% isopropanol with sufficient cHCl to make the solution 6N with respect to HCl. The chromatogram was run for 48 hours at 30°C. after which it was dried and the UV absorbing areas determined.

4. Ion Exchange Chromatography

(a) Amino Acids

Large scale separation of amino acids was attempted using the technique of Moore and Stein (1958).

Rexyn 101 (Fisher Scientific), a sulphonated polystyrene copolymer, was washed with 2 litres of 0.14N HCl, followed by 500 ml. of distilled water. The resin was then suspended in 2 litres of 2N NaOH and heated for 1 hour on a water bath. It was then washed with distilled water until the pH of the effluent was 7.0

A slurry was made of the resin with the initial buffer to be used and two columns were packed according to the procedure of Moore and Stein. One column was 150 x 0.9 cm. and

the second 15 x 0.9 cm. The columns were prepared by washing with 0.2N NaOH, followed by the appropriate buffer - 0.2N sodium citrate buffer, pH 3.25 for the 150 cm. column and 0.2N sodium citrate buffer, pH 5.28, for the 15 cm. column.

The amino acid sample was introduced into the 150 cm. column, followed by a flow of 0.2N sodium citrate buffer at pH 3.25. The flow rate was adjusted to about 15 ml./hour by means of slight air pressure at the top of the column. Temperature was maintained at 37°C.

After 150 fractions of 1.5 ml. each had been collected, a 0.2N sodium citrate buffer at pH 4.25 was introduced, and a further 150 fractions were collected with a fraction collector. In this manner, the acidic and neutral amino acids were eluted from the column. The column was then re-equilibrated with 0.2N NaOH.

The procedure with the 15 cm. column was essentially the same but the initial and only buffer used as an eluent was 0.2N sodium citrate buffer, pH 5.28, and the temperature of the column was 50°C. Seventy-five fractions of 1.5 ml. were collected to elute the basic amino acids and the column was re-equilibrated.

The presence of amino acid in each fraction was determined by spotting one drop of the fraction onto filter paper and spraying with ninhydrin.

(b) Nucleotides

The procedures of Cohn (1950), and Cohn, Volkin and Khym (1951) were employed for the large scale separation of nucleotides.

A column of Rexyn AG1 (Fisher Scientific), a polystyrene alkyl quaternary amine of the chloride-sulphate form was constructed by pouring a slurry of resin and water in a column to a height of 5 cm. Equilibration of the column was accomplished by washing with first, 10 column volumes (CV) of 1.0N NaOH, then 2 CV of 1.0M NH_4Cl, 10 CV of 1.0N HCl and finally sufficient distilled water to raise the effluent flow to a pH greater than 3.

The sample was prepared for the column by the addition of M NH_4OH until the pH of the sample was above 8. It was then diluted to a total anion (including nucleotides) concentration of 0.01M or less.

Absorption onto the column was accomplished at a flow rate of not more than 3 ml./cm^2/minute. After absorption of the sample, the column was washed with 2 CV of water, then enough 0.01M NH_4Cl to lower the effluent pH to below 7.

Elution of the column was initiated with 0.002N HCl. The absorption at 260 mμ of the 5 ml. fractions collected were observed and when the OD dropped to below 0.01, 0.005N HCl was flowed through. This eluent was continued until no further UV absorbing material was apparent in the effluent fractions.

5. Isotopic Enrichment of the Medium

Sufficient $N^{15}H_4Cl$ was added to 24 hour cultures to attain a 1% proportion of heavy isotope in relation to the total ammonium chloride in the medium. The cultures consisted of 20 ml. of synthetic medium inoculated with Micrococcus

sodonensis and were agitated vigorously at room temperature for 24 hours before enrichment. Enriched cultures were allowed to incubate for designated periods of time, after which the flasks were removed from the agitator and immersed in a dry ice-acetone mixture to inhibit further metabolism. The same procedure was employed with non-enriched cultures which served as controls. The cells were washed three times at 6°C. in distilled water and the various extraction procedures were carried out with the cell pellets.

Experimental and Results

1. Identification of Extracts

Analysis of the boiling water extract of 48 hour cells grown in basal medium + NH_4^+, by the procedure of Rockland and Underwood, showed it to contain 6 amino acids. After hydrolysis of the extract with 6N HCl for 12 hours at 121°C. in an autoclave, no increase in amino acids was found.

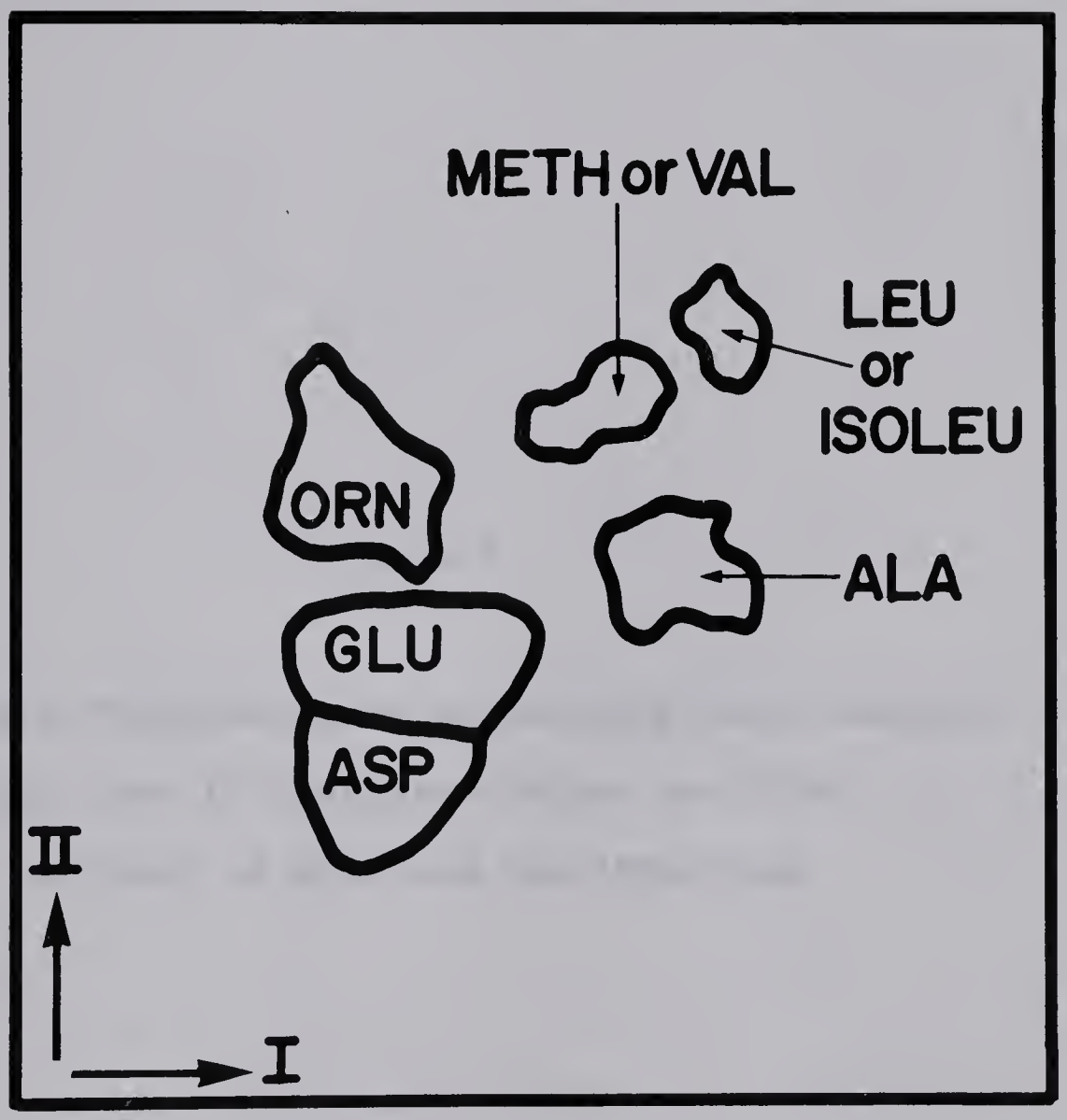
Confirmation of the pool's constituents was made by the single phase, descending chromatography system. Seventeen known amino acids were run as standards. The pool was shown to be mainly glutamic and aspartic acids, with some ornithine and trace amounts of alanine, methionine or valine, and leucine or isoleucine. An exact quantitation of the pool was not undertaken. (See Figures 8 and 9).

Paper chromatography and spectrophotometry showed the cold TCA extract to be the 5' mononucleotides (see Figure 10 and 11). If any other components were present in the pool, they were in such small amounts as to be undetectable by these techniques. The largest single component was adenosine-5'-monophosphate (AMP). Figure 10 represents two separate chromatograms run at different rates in which the components of the CAP of 48 hour old cells, grown in basal medium + NH_4^+ are compared with four sets of standards in known amounts. Figure 11 is the UV scan of the 4 spots separated by chromatography. These were eluted in 0.07M phosphate buffer at pH 7 and the pH was adjusted to 1 with cHCl before scanning between 200 and 350 mμ with a recording spectrophotometer.

Figure 8

Two-dimensional Chromatography of Boiling Water Extract (AAP)
by the Procedure of Rockland and Underwood

FIGURE 8




I
II

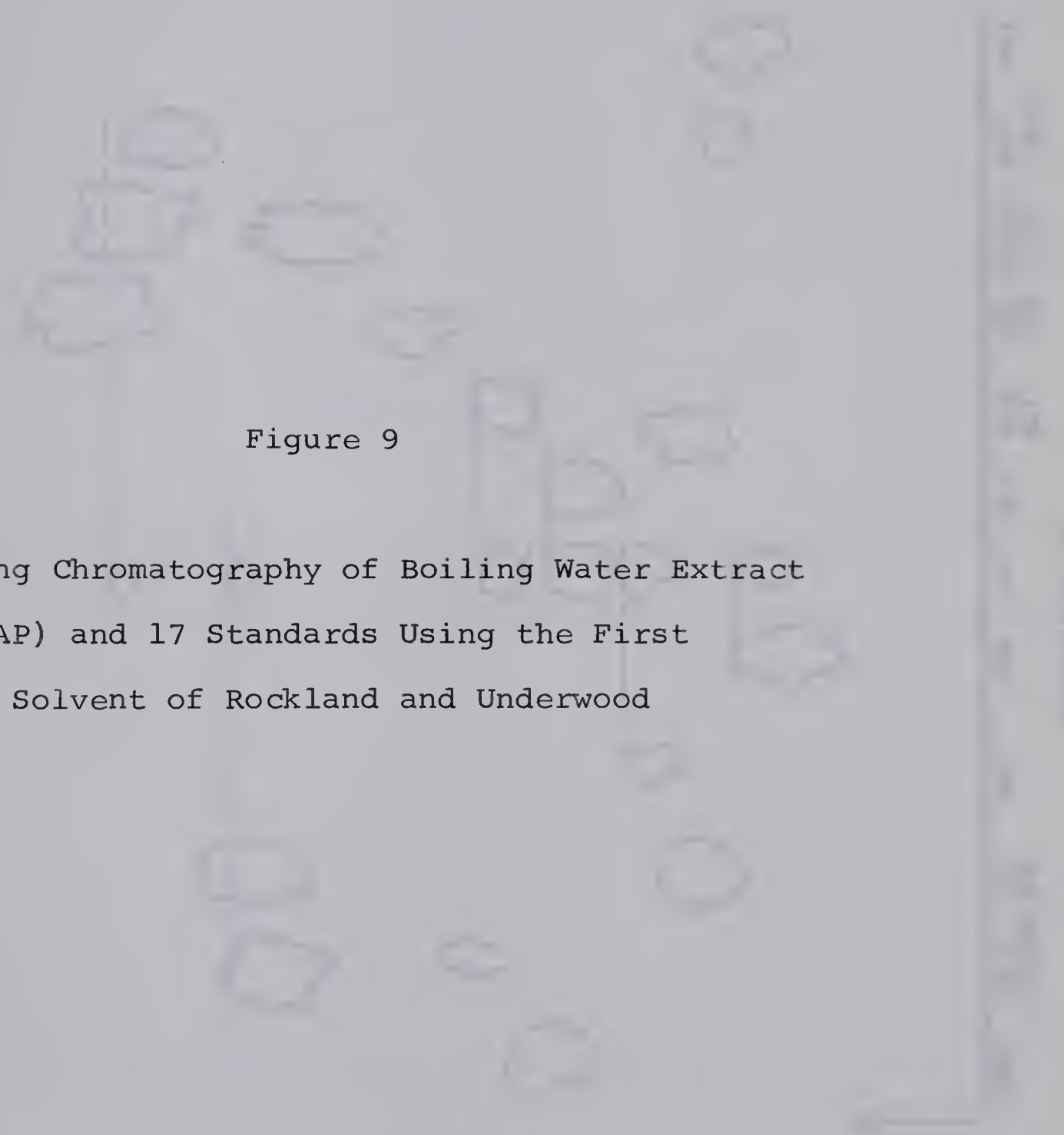


Figure 9

Descending Chromatography of Boiling Water Extract (AAP) and 17 Standards Using the First Solvent of Rockland and Underwood

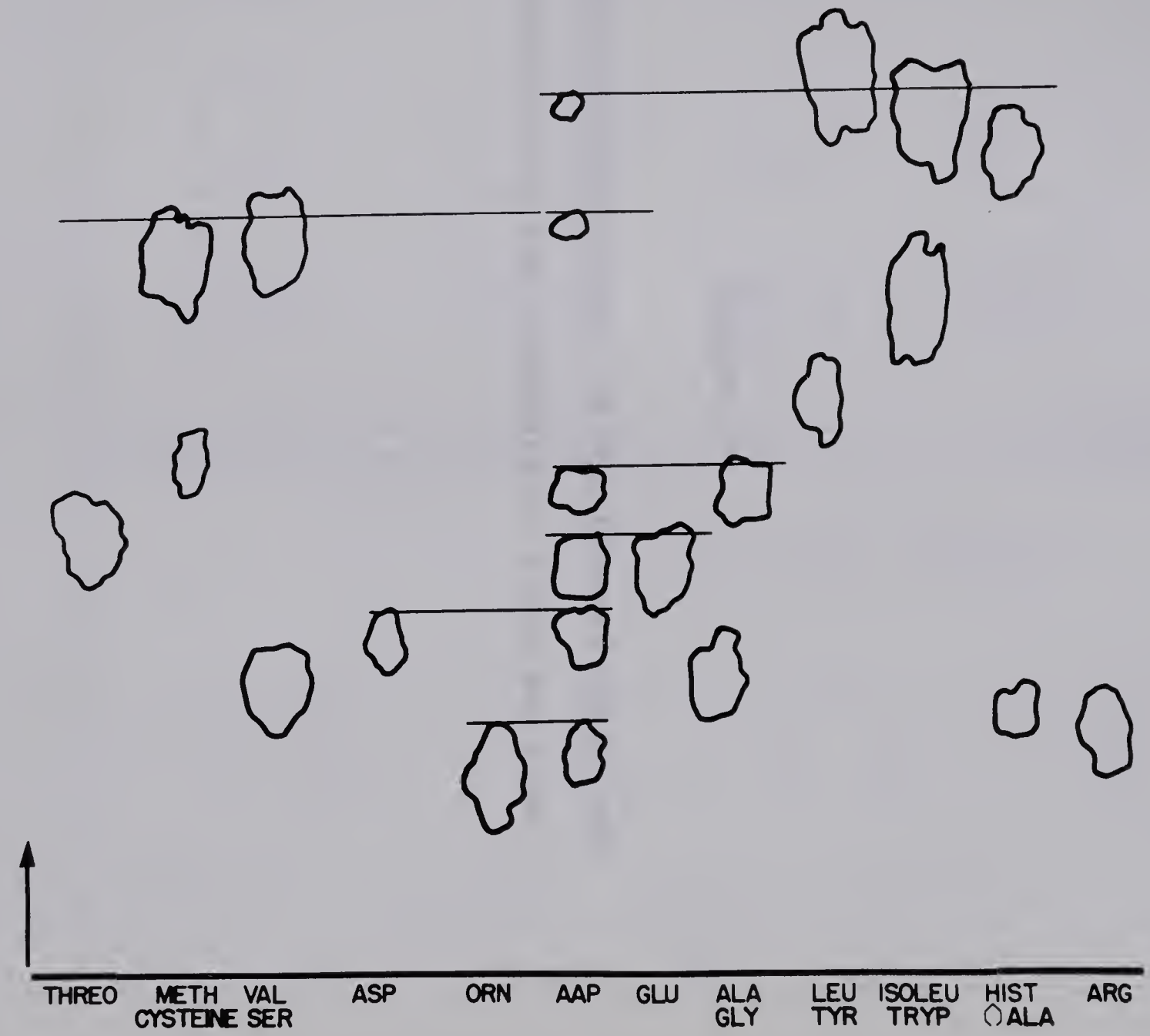
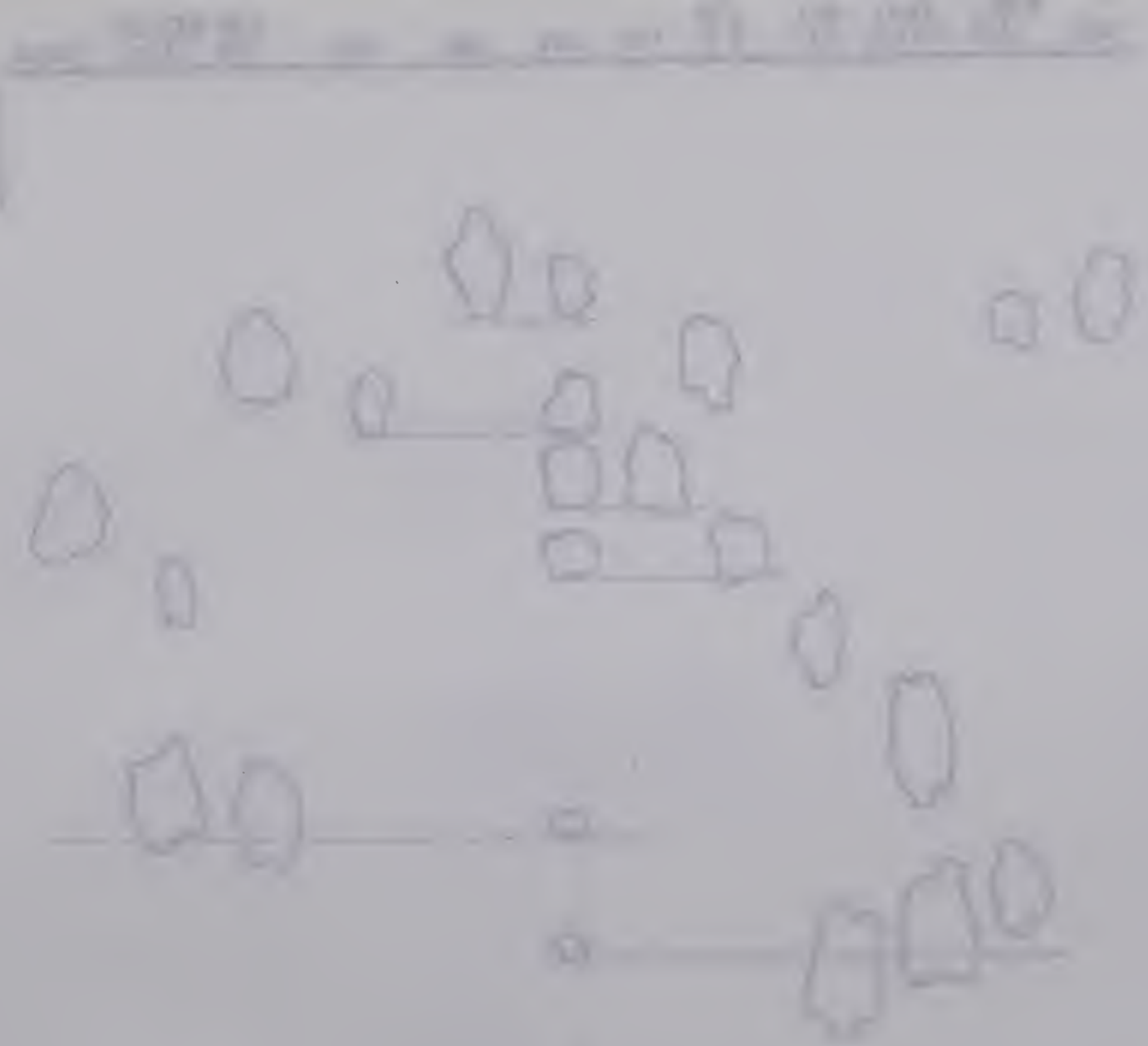

THREO
METH
CYSTEINE
VAL
SER
ASP
ORN
AAP
GLU
ALA
GLY
LEU
TYR
ISOLEU
TRYP
HIST
ALA
ARG

FIGURE 9



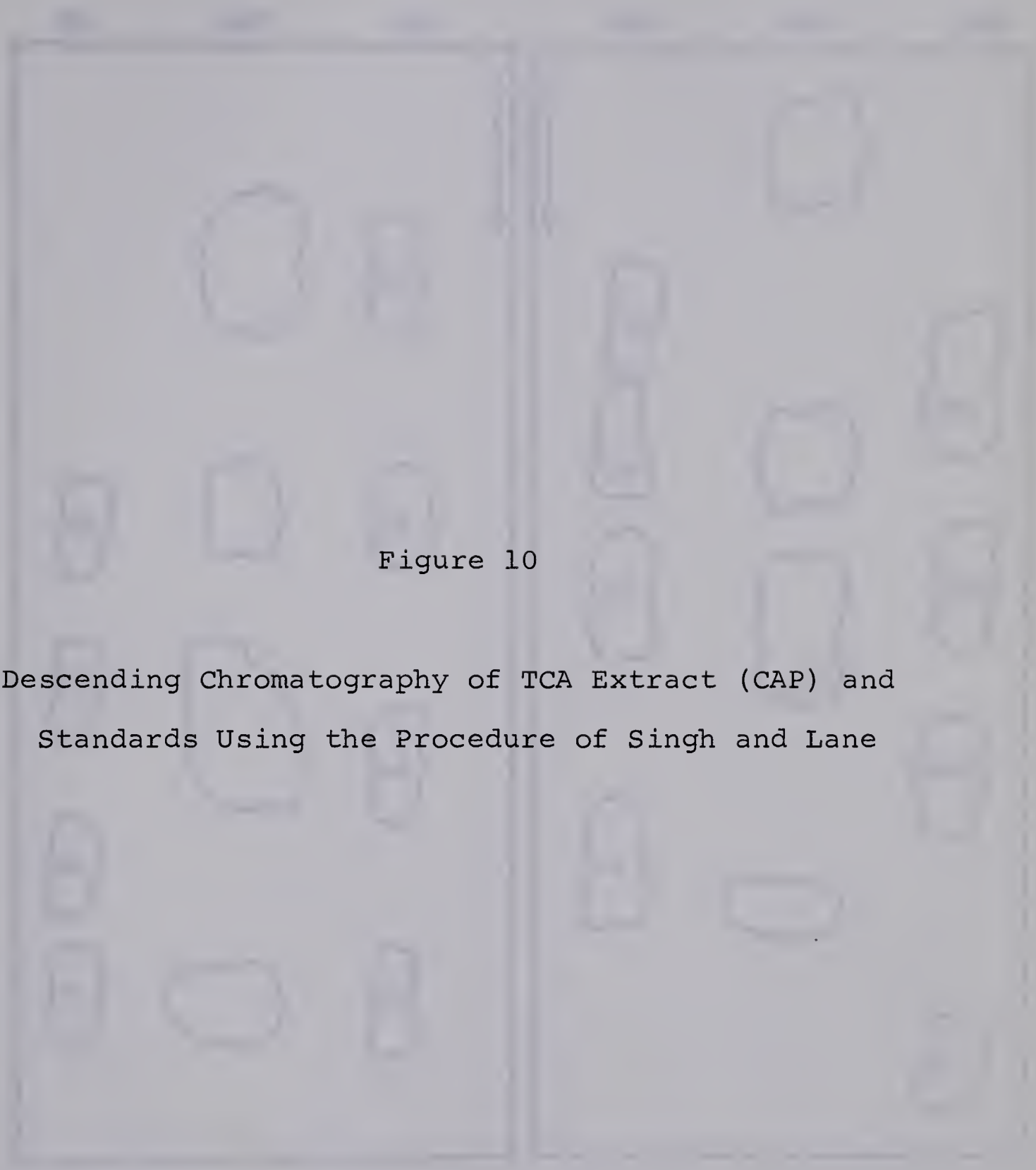
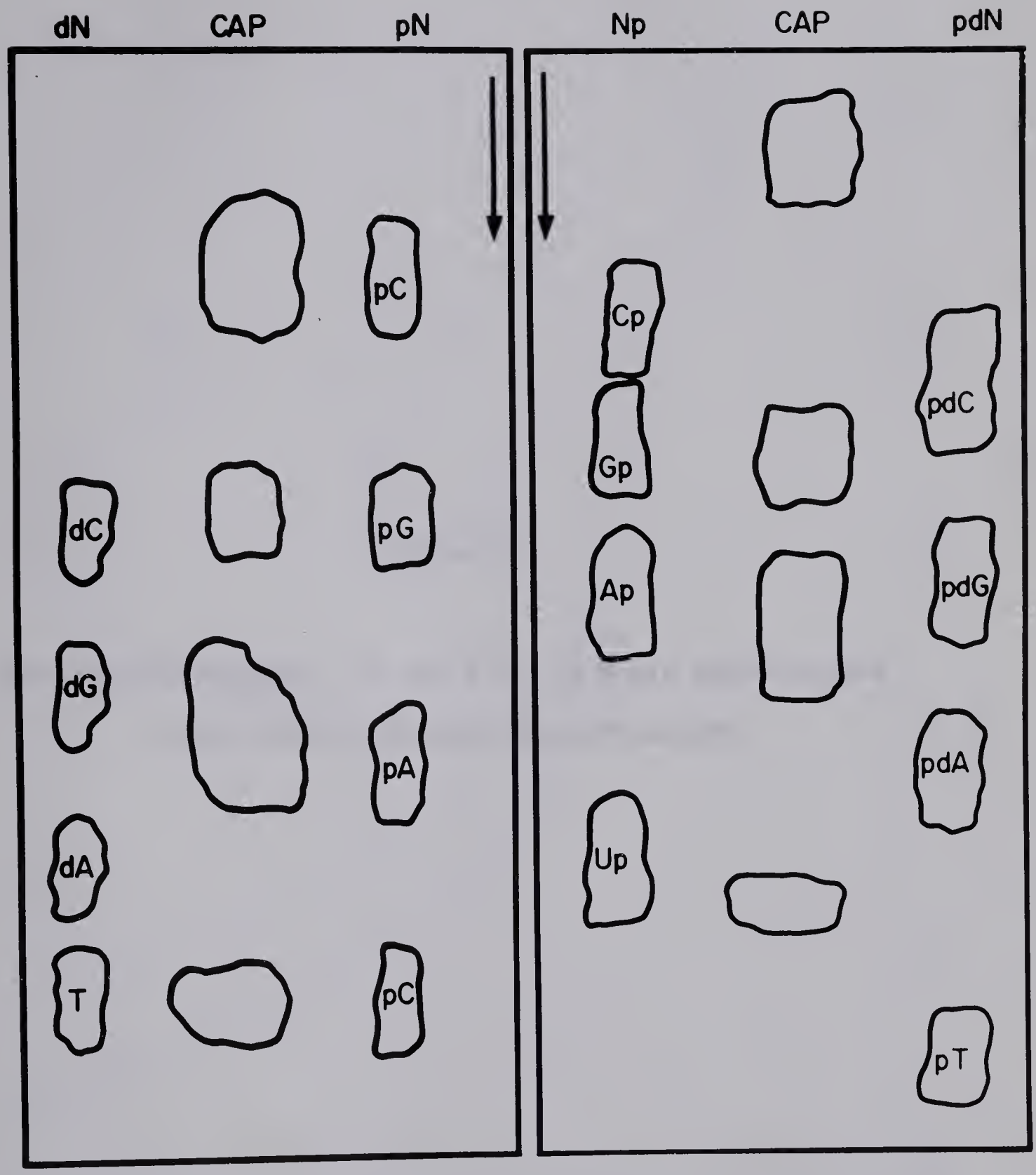


Figure 10

Descending Chromatography of TCA Extract (CAP) and Standards Using the Procedure of Singh and Lane

FIGURE 10



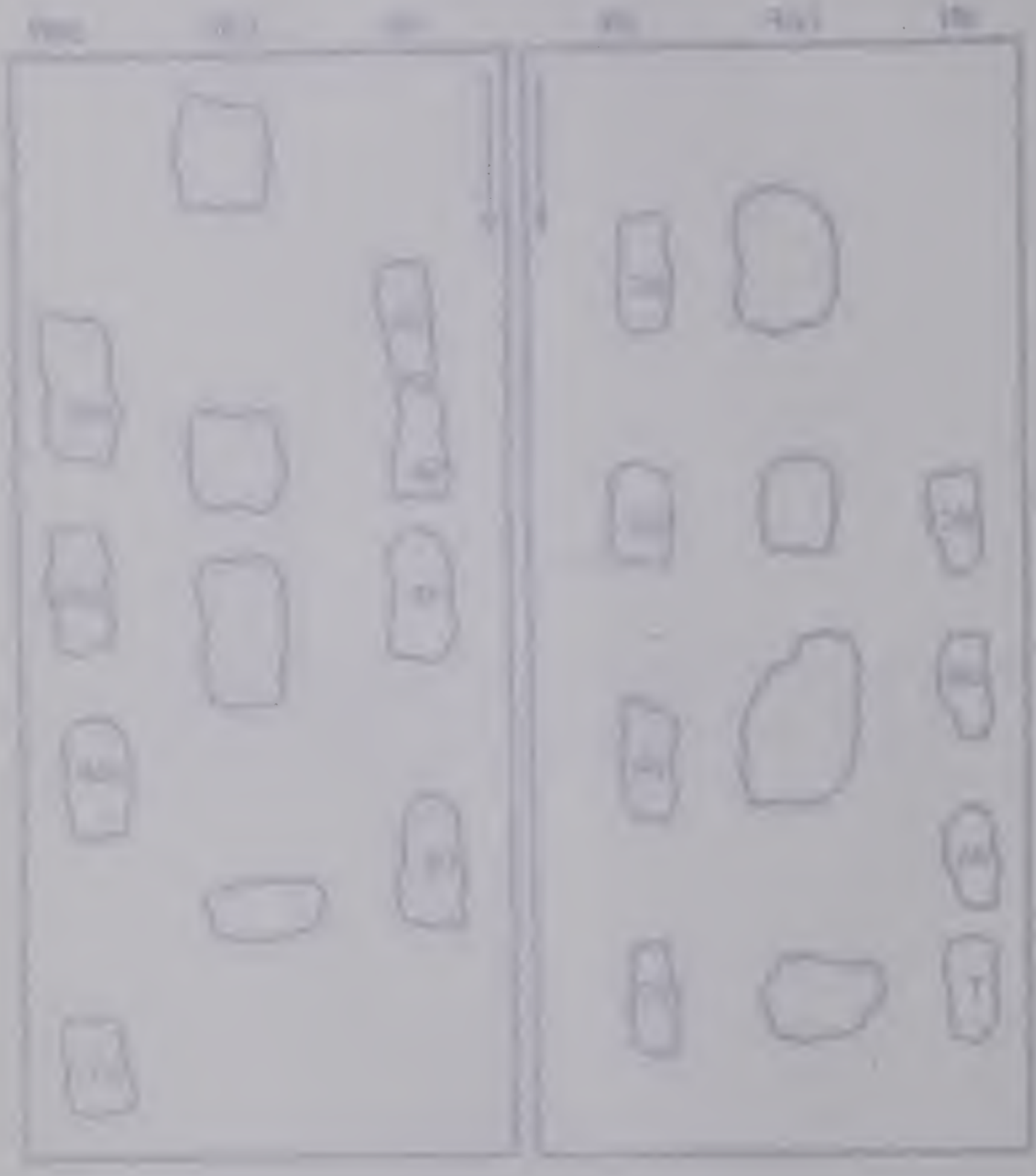


Figure 11

Absorption Spectra, at pH 2.0, of Four Nucleotides
Separated by Paper Chromatography

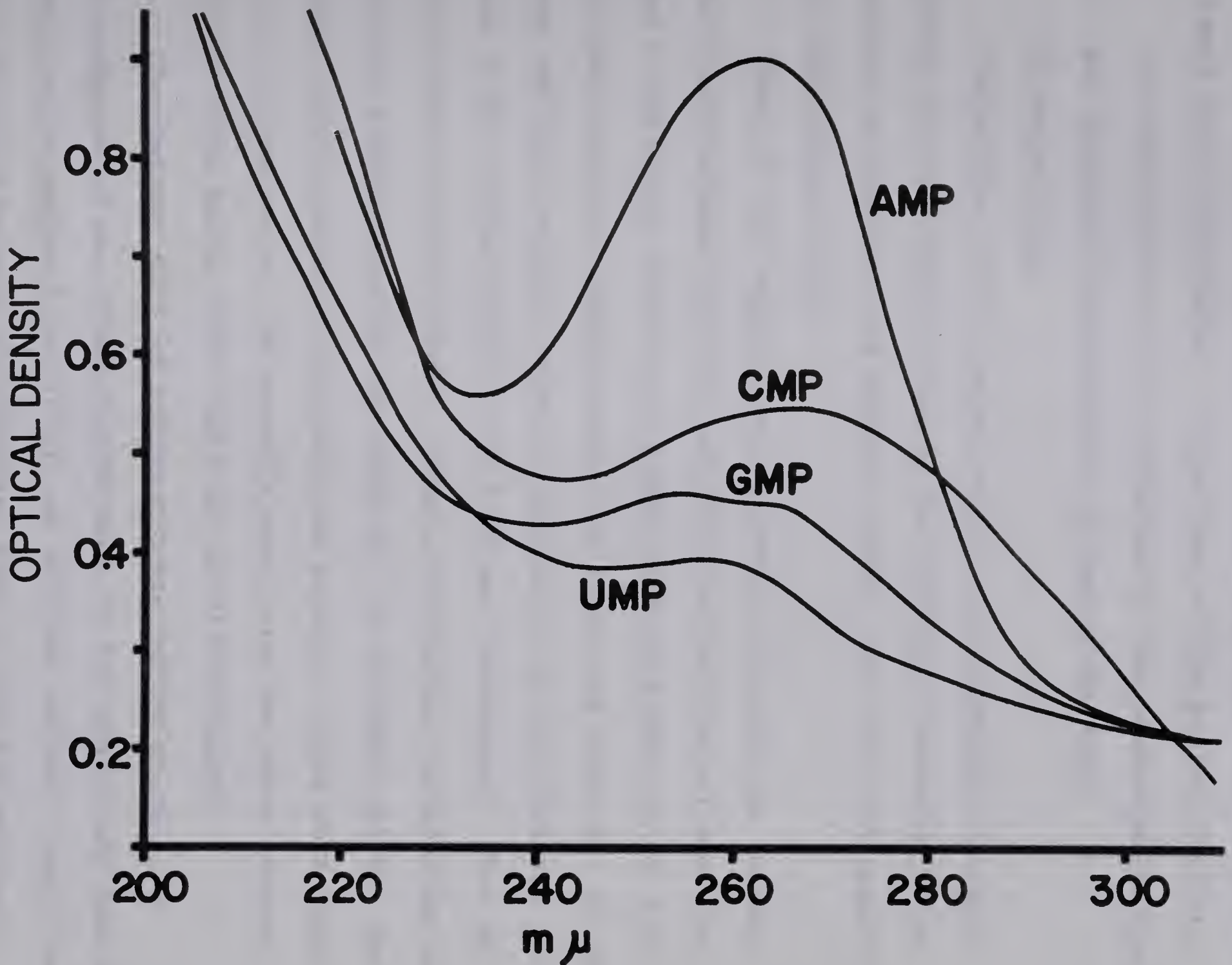
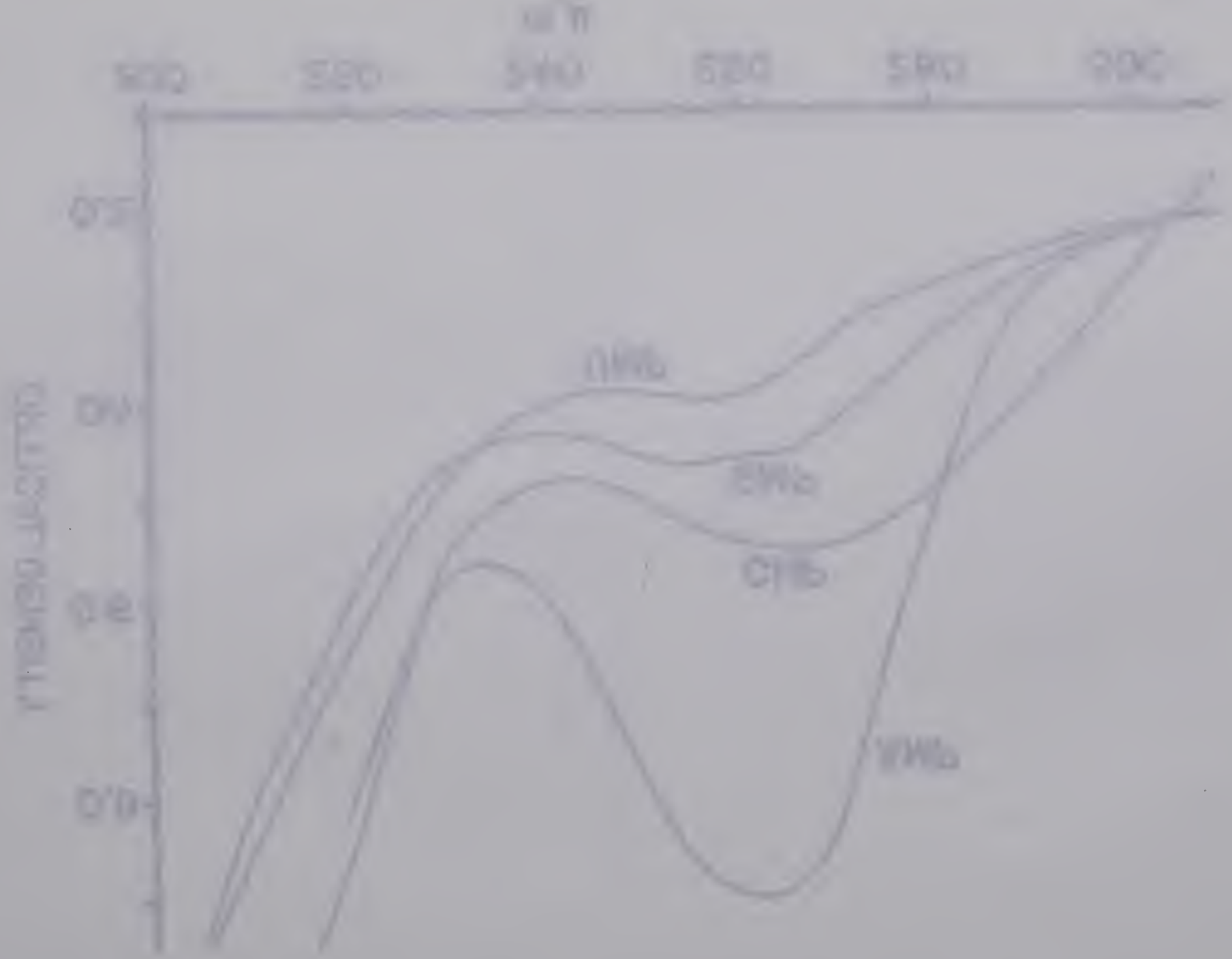

OPTICAL DENSITY
0.8
0.6
0.4
0.2
AMP
CMP
GMP
UMP
200
220
240
260
280
300
mμ

FIGURE II


T, °C
500
520
540
560
580
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620
640
660
680
700
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780
800
820
840
860
880
900
920
940
960
980
1000
1020
1040
1060
1080
1100
1120
1140
1160
1180
1200
1220
1240
1260
1280

The wavelengths at which maximum and minimum absorbance occurred in the four scans corresponds to those found in published data (Calbiochem - Properties of the Nucleic Acids).

No free bases could be detected by the technique of Wyatt.

2. Ion Exchange Chromatography of Extracted Pools

(a) Amino Acids

Separation of the amino acids by ion-exchange chromatography was not feasible due to the disproportionately large amounts of glutamic and aspartic acids, and the trace quantities of other amino acids.

Most of the aspartic acid passed in the 150 cm. column was found between the 80th and 120th ml. of the effluent volume - the eluent being 0.2N sodium citrate buffer at pH 3.25. Glutamic acid was found between the 150th and 200th ml. of the same effluent volume. After a further 25 ml. of effluent volume, 0.2N citrate buffer at pH 4.25 was introduced. This pH should have eluted alanine, valine, methionine, isoleucine and leucine respectively by the 450th ml. of effluent volume. However, trace amounts of glutamic and aspartic acids were found in all fractions of the second eluent, and the trace amounts of the above amino acids could not be distinguished.

The passage of 0.2N sodium citrate buffer at pH 5.28 through the 15 cm. column eluted most of the acidic and neutral amino acids in the first 10 ml. of effluent volume. Ornithine was recovered between the 35th and 50th ml. of the effluent volume. Traces of glutamic acid and aspartic acids were found in all fractions of this eluent.

In the operation of the columns, Moore and Stein proposed 1.0 μM amounts of all amino acids. This was not possible with the boiling water extract. Glutamic and aspartic acids were far in excess of the recommended amount. The poor quality of eluting resolution was also attributed to the 37°C. temperature in which the 150 cm. column was operated. The recommended temperature was 50°C.

(b) Nucleotides

A known mixture of four nucleotides was separated by the technique of Cohn et al. Employing 0.002N HCl as the eluent, the first 30 ml. of the effluent volume contained cytidylic acid. This was followed by adenylic acid between the 50th and 80th ml. of the effluent volume. No further UV absorbing material could be determined after 120 ml. of effluent volume. The 0.005N HCl effluent was initiated, and uridylic acid was eluted after 100 ml. of eluent has passed through the column. Guanylic acid began to appear after 150 ml. of the effluent volume and could no longer be discerned after 250 ml. had passed through the column.

The constituents of the TCA extract were not easily discerned by this method. Only by evaporating the effluent volume and resuspending the residue in 5 ml. of distilled water could an increase in UV absorbance be seen. Forty ml. of a 24 hour culture were employed for the extraction, and the total absorbance was less than 0.10 in a volume of 5 ml.

3. Isotopic Enrichment

In early experiments with the heavy nitrogen isotope, the proportion of $N^{15}H_4Cl$ was 1.0%. Twenty gaseous samples,

ranging from 2 to 50 minutes, were prepared from cultures having this concentration. No reliable figures for enrichment were obtained using this concentration, since the natural abundance of N^{15} is approximately 0.35%.

Eleven mg. of $N^{15}H_4Cl$, containing 96.7% N^{15} (Merck), were added to 20 ml. cultures of Micrococcus sodonensis to make the final proportion of the heavy isotope 50% of the nitrogen of ammonium chloride in the medium. Duplicate, non-enriched cultures, to which 11 mg. of NH_4Cl had been added, served as controls. Cultures were allowed to incubate for periods of time from 30 seconds to 50 minutes, after addition of the salt, before interrupting metabolism.

The boiling water and TCA extracts were taken from the same cultures. Separate 20 ml. cultures were used for the extraction of nucleic acids. The amount of DNA extracted from a 20 ml. culture was considered too small to be practical and was combined with the RNA fraction for enrichment studies.

Several problems were encountered in the formation of nitrogen samples. The steam-distilled product was added to the reaction flask, and evacuation was commenced. To ensure a vacuum of less than 1×10^{-5}, the apparatus shown in Figure 7 was pumped down for 30 minutes in all cases. The addition of NaOBr resulted in the oxidation of ammonium salts and the methyl red indicator present in the solution. The ethanolic base of the indicator presented the problem of probable CO_2 evolution. In addition, some O_2 was formed from the decay of NaOBr. The entry of CO_2, H_2O, and O_2 into the mass spectrometer resulted in CO formation upon contact with the

filament. Any carbon source in the spectrometer enhanced this condition. CO formed by these sources contributed to the mass 28 peak and therefore decreased the true value of N^{15} enrichment. Atmospheric contamination, having CO_2, H_2O, O_2 and a large percentage of $N^{14}N^{14}$, also resulted in a lower enrichment value. Most of these sources of contamination were overcome by freezing the reaction flask after oxidation. Liquid air, at approximately -190°C., froze all components of the reaction flask except nitrogen gas. This entered the breakseal, and after allowing several minutes for equilibration of the gas, the stopcock was closed and the breakseal flamed off. In the event of atmospheric contamination through improper sealing, enough of the gaseous sample was present in the reaction flask to allow equilibration with a second breakseal. As a further precaution, the breakseal was immersed in liquid air before entry of the sample into the mass spectrometer.

These precautionary measures did not prevent contamination by atmospheric nitrogen and samples with unexpectedly low enrichment were attributed to this.

The possibility of excessive enrichment was neglected since the condition could arise only through improper washing of the cells or small memory effects in the Kjeldahl apparatus. The second possibility was minimized by washing the apparatus between samples.

The recommended size of sample for analysis is one mg. of gaseous nitrogen. In instances where the amount of sample was insufficient, the readings obtained were considered unreliable. The residual measurements of the analyser

were usually about 0.42 At. % N^{15}, and the addition of a small sample did not increase the amount of pressure in the analyser appreciably. Consequently, a low enrichment reading for a sample only slightly larger than the residual gases was considered to be in error. Contamination by atmospheric gases was measured by observing the argon peak at mass 40.

Although 4 series of experiments were undertaken, only the latter two were considered reliable. As mentioned before, the proportion of enrichment in the first series was too low and could not be distinguished from variation arising in the measurement of the absolute N^{14}/N^{15} ratios. Many of the samples in the second series had a low volume of gas because the cultures employed were only 18 hours old, thereby decreasing the amount of extractable material. The results of two sets of experiments, designated chi (χ) and delta (Δ) series, are shown in Table 10 and Figure 12. In most cases, the absence of a value on the table indicates a contaminated sample - usually the presence of atmospheric gases as a result of leakage. In both series, a 50% enrichment was employed.

In both series, an equilibrium was established by 30 seconds in the early nucleotide pool at approximately 0.9%. This value was maintained throughout the time intervals of the two experiments. The uniformity of rate seen in the nucleotide pool could be a result of the proposed requirement for NH_4^+ in the synthesis of purines and pyrimidines. If any variation in the nucleotide pool did exist between the two experiments, the equilibriums were established too rapidly to detect any variation in rate. Experimental time

Table 10

Mass Spectrometric Analysis of the Amino Acid and Early Nucleotide Pools of Two Experiments

Time (min. after culture enrichment)	At. % N^{15} Nucleotide Pool Series χ	Nucleotide Pool Series Δ	Amino Acid Pool Series χ	Amino Acid Pool Series Δ
1/2		0.8436		4.7379
1		0.7449		5.7843
2	0.8203			
3		0.9169		
4	0.9049		1.8372	
6	0.9616			
7				6.605
8	0.7559	0.6489	2.4253	5.7524
10		0.7490	3.3001	6.6831
20	0.7216		5.3140	
50			3.7200	
Control	0.4240			

Figure 12

Enrichment of Pools Plotted Against Time

in Two Series of Experiments

aap - Amino Acid Pool

cap - Early Nucleotide Pool

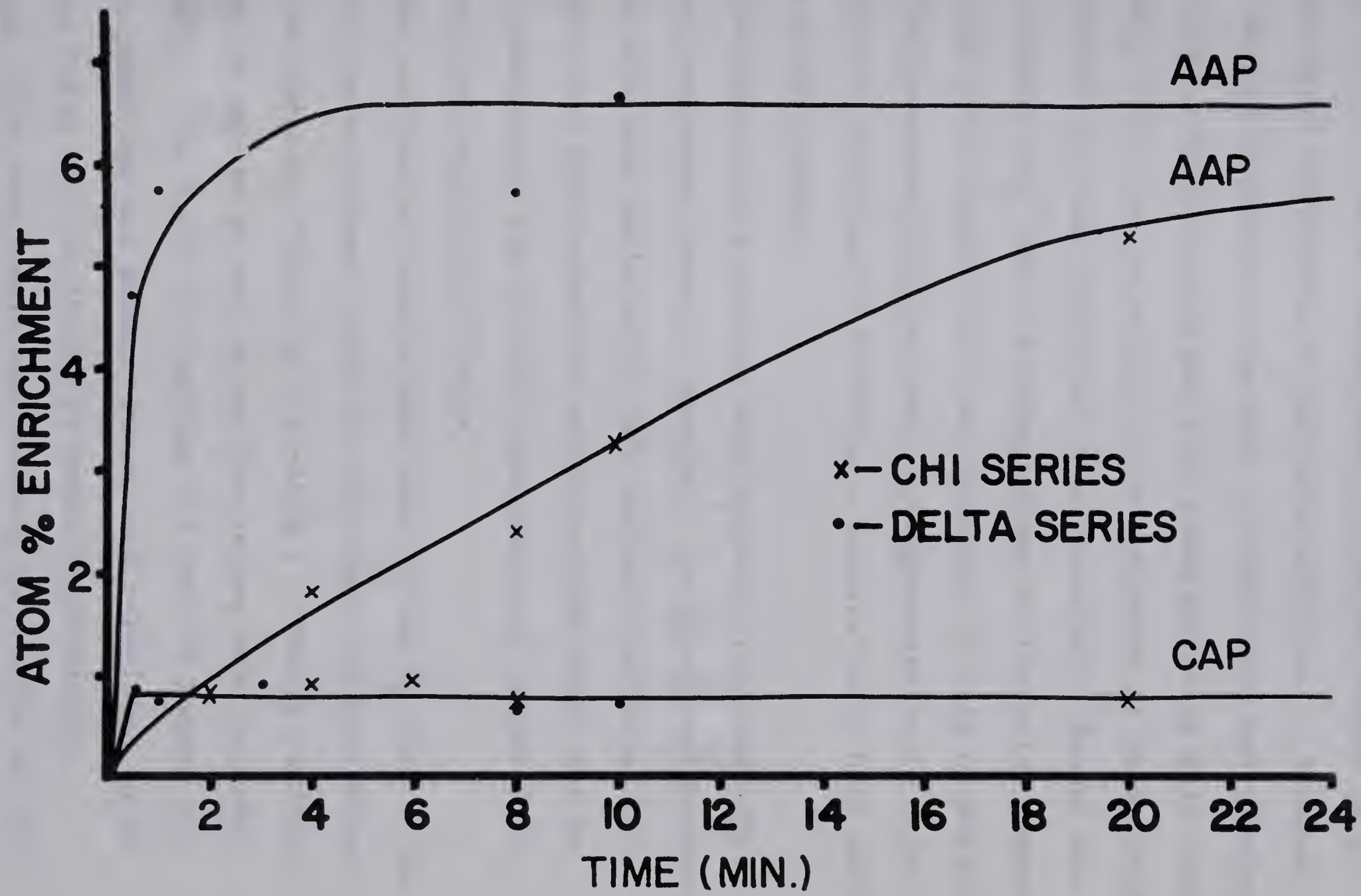

AAP
AAP
x—CHI SERIES
•—DELTA SERIES
CAP
ATOM % ENRICHMENT
6
4
2
2
4
6
8
10
12
14
16
18
20
22
24
TIME (MIN.)

FIGURE 12

RAA
PAA
PAC
CALCULATED AT DEPTH OF 1.5 CM
TEMPERATURE INCREMENT

intervals of less than 30 seconds were considered impractical.

An equilibrium of approximately 6.5% enrichment in N^{15} was found after 20 minutes in the amino acid pool. However, the two series differed in the rate at which this equilibrium was attained. Many factors may have contributed to this variation. NH_4^+ would not be vital to the synthesis of amino acids when glutamic acid was available as a precursor. Random exchanges between NH_4^+ and the amino groups of the acids present in the pool could account for the variation in rate of enrichment. Environmental factors such as temperature would also contribute to variation in reaching a comparatively high equilibrium of enrichment.

Studies with the nucleic acid extracts (DNA and RNA) showed no enrichment after 10 minutes. Enriched nucleotides from the cell pool would enter into polymer synthesis, but enrichment in a high molecular weight polymer would not be evident immediately and further experiments of longer duration were considered unprofitable.

The analyses of samples were exemplified by that calculated for the enrichment found in the amino acid pool after 10 minutes duration (Table 11 - 10-AAPΔ). Twenty readings were made of masses 28 and 29 and the values were recorded graphically. The first 11 readings are seen in Figure 13. Residual peaks are not shown. These were calculated to be 42.62 and 1.244 for the 28 and 29 peaks respectively.

Mass 28 of the sample was measured on a scale of 3000 millivolts (mv) and the mass 29 on a scale of 300 mv. The

average reading of 5.55 for mass 28 indicates 5.55/10 of 3000 mv. or 1665 mv. From this was subtracted the average value of the residual readings. The same procedure was applied to mass 29 readings. Sequential pairs of both masses were averaged and the 10 ratios of these values were averaged to calculate an R value of 8.9815. This was applied to the equation of Martin et al to yield a value of 6.6831 At.% N^{15}.

The determination of enriched components of the amino acid pool was impossible due to the problems experienced in the large scale separation of the pool by ion-exchange chromatography.

Although a satisfactory procedure for the separation of the nucleotide pool was obtained, the amounts of each constituent were too small to employ mass spectrometric analysis. Further experiments with cultures of up to 2 litres would be necessary to determine the atom % N^{15} of various nucleotides in the pool.

Table 11

Enrichment Calculation of Sample 10 - AAP△

Residual readings

28 on 100	x 100/10	29 on 10	x 10/10
4.25	42.5	1.30	1.30
4.25	42.5	1.25	1.25
4.27	42.7	1.25	1.25
4.27	42.7	1.22	1.22
4.27	42.7	1.20	1.20
	42.62 av.		1.244 av.

Sample readings

28 on 3000	x3000/10	-42.62	R values 28 av./29 av.	-1.244	x300/10	29 on 300
5.70	1710.00	1667.38	1664.38/238.906	239.056	240.300	8.01
5.68	1704.00	1661.38		238.756	240.300	8.00
5.68	1704.00	1661.38	1661.88/237.106	238.156	239.400	7.98
5.65	1695.00	1652.38		236.056	237.300	7.91
5.62	1686.00	1643.38	1640.38/235.456	235.756	237.000	7.90
5.60	1680.00	1637.38		235.156	236.400	7.88
5.60	1680.00	1637.38	1634.38/233.806	234.556	235.800	7.86
5.58	1674.00	1631.38		233.056	234.300	7.81
5.55	1665.00	1622.38	1620.88/232.006	232.756	234.000	7.80
5.54	1662.00	1619.38		231.256	232.500	7.75
5.51	1653.00	1610.38	1608.88/229.606	230.956	232.200	7.74
5.50	1650.00	1607.38		228.256	229.500	7.65
5.47	1641.00	1598.38	1593.88/228.556	229.756	231.000	7.70
5.44	1632.00	1589.38		227.356	228.600	7.62
5.40	1620.00	1577.38	1577.38/226.006	226.756	228.000	7.60
5.40	1620.00	1577.38		225.256	226.500	7.55
5.35	1605.00	1562.38	1560.88/223.756	224.356	225.600	7.52
5.34	1602.00	1559.38		223.156	224.400	7.48
5.31	1593.00	1550.38	1548.88/222.406	223.456	224.700	7.49
5.30	1590.00	1547.38		221.356	222.600	7.42

R values

6.966
7.009
6.966
6.990
6.986
7.007
6.973
6.979
6.975
6.964

6.9815 = averaged R

$$\frac{100}{2R + 1} = 6.68 \pm 0.015$$

atom percent enrichment of N^{15} in 10 - AAP△

Figure 13

Mass Spectrogram of 10 - AAP△

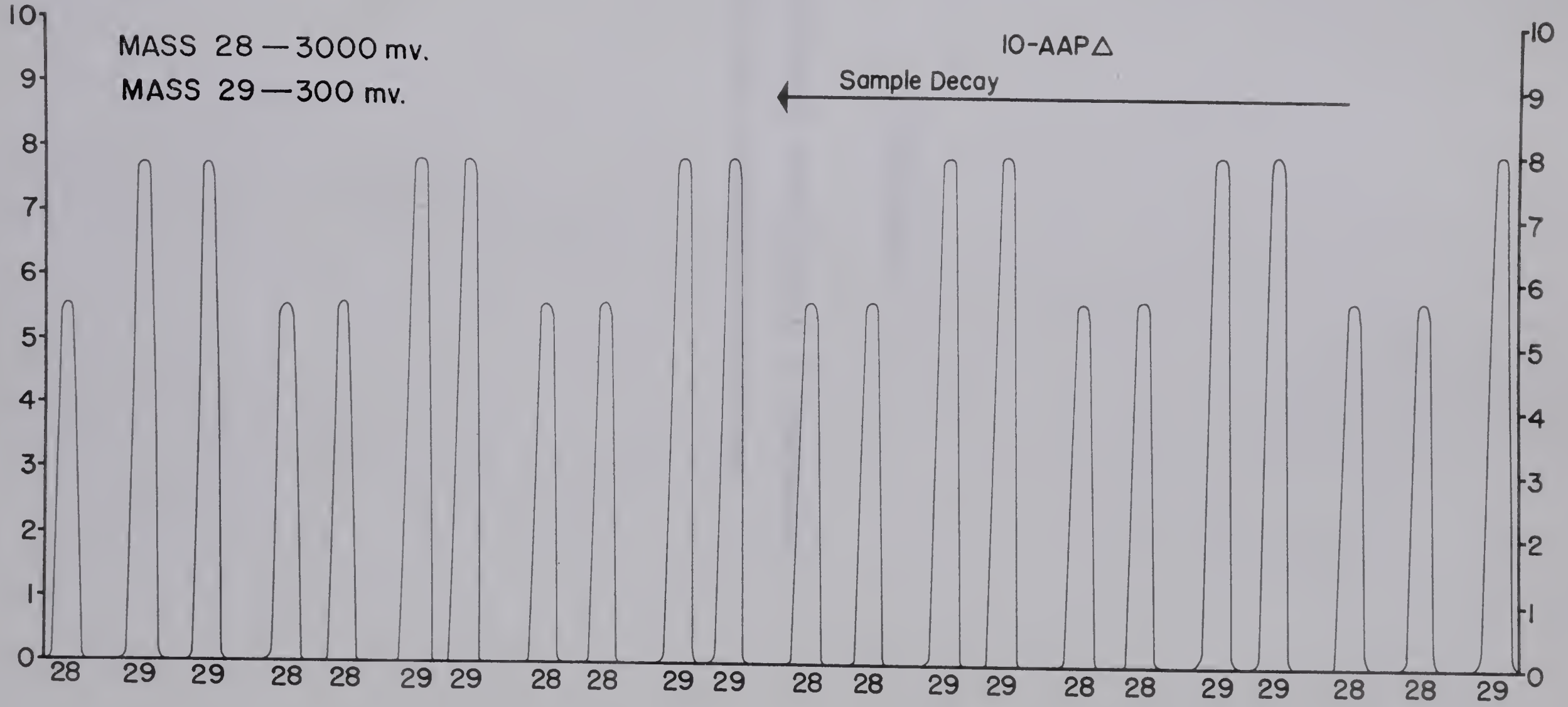
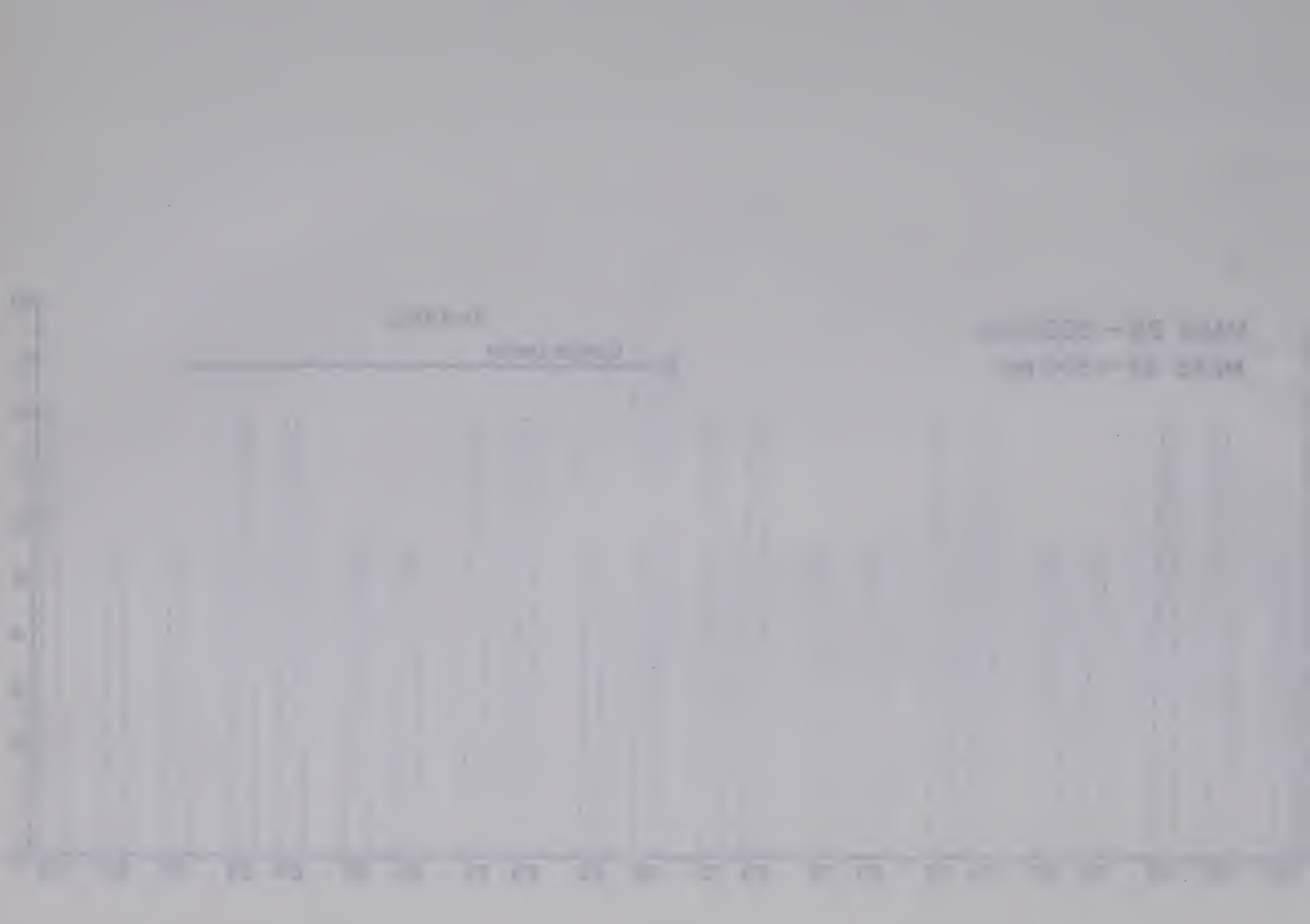

MASS 28 — 3000 mv.
MASS 29 — 300 mv.
10-AAP△
Sample Decay
10
9
8
7
6
5
4
3
2
1
0
28 29 29 28 28 29 29 28 28 29 29 28 28 29 29 28 28 29 29 28 28 29

FIGURE 13



DISCUSSION

(Refer to metabolic chart at end of discussion for structural formulae and reactions)

Discussion

The maximum amount of NH_4^+ uptake by Micrococcus sodonensis was seen in the first 12 hours of growth. An average value of 3.7 μM was determined for this period. Up to 12 hours, there was a fairly steady decrease of NH_4^+ in the medium. After 12 hours, there was a gradual augmentation up to 30 hours, after which the concentration remained constant to the end of the experiment. It is easily discerned from these findings that a definite requirement for NH_4^+ exists in a young culture of Micrococcus sodonensis. As the population entered the logarithmic growth phase, the rate of NH_4^+ uptake was less easily determined because of NH_4^+ released by deamination of amino acids, in particular glutamic acid, and the autolysis of cells.

Although the experimental conditions of Zarlengo and Abrams (1963) differed in some respects to those in the present report, some interesting comparisons are evident. In their experiments, aged cultures of Streptococcus faecalis were observed with respect to NH_4^+ uptake. The aged cultures were retarded in glycolysis as a result of a decrease in internal pH. The situation was reversed by the addition of NH_4^+. One ml. of cells suspended in a NH_4Cl solution at pH 7 took up about 4 μM of NH_4^+ in 25 hours. This increased slowly to 4.5 μM by 36 hours. Their findings indicate that free NH_3 rapidly equilibrates across the cell membrane by simple diffusion while H^+ and NH_4^+ remain comparatively impermeable. Once across the membrane, NH_3 is converted to NH_4^+ which is passively accumulated to a concentration greater than that present in the

medium. The lower intracellular pH constitutes the driving force for this accumulation of NH_4^+ against a concentration gradient.

In Micrococcus sodonensis, by contrast, intracellular NH_4^+ is rapidly incorporated into organic compounds. Experiments with various energy sources indicate that NH_4^+ plays no role in the energy metabolism of Micrococcus sodonensis. Lactic acid, which is employed in the synthetic medium, had the most notable effect as an energy source. Complex carbohydrates would not support the growth of the organism to any extent. Sucrose, glucose, ethanol and maltose functioned as energy sources to a lesser extent than did lactic acid.

The substitution of nucleic acid derivatives for NH_4^+ resulted in a duplication of the NH_4^+ effect in some cases. Adenine, adenosine and adenosine phosphates were distinctly inhibitory to the growth of the organism. The determination of the rate of growth response to adenosine showed that the inhibition was eventually overcome to some extent by the addition of NH_4^+.

The growth response to cytosine, uracil and uridine were similar to that obtained with NH_4^+ and the inclusion of NH_4^+ in the medium resulted in greater growth in some cases.

The response to guanosine was 33% greater than that obtained with NH_4^+. The addition of NH_4^+ to guanosine did not change the response after 48 hours. The increased response to guanosine was shown to lag initially. This may indicate an induced enzyme system in the transport of this

nucleoside. However, once incorporated into the cell, guanosine appeared to function well in place of NH_4^+.

NH_4^+ functions as the amino donor in the formation of GMP from XMP (Lieberman - 1956). In the absence of NH_4^+, the synthesis of this essential nucleic acid component would be effectively inhibited. A similar situation exists in the case of uridine and cytidine which are synthesized by the incorporation of NH_4^+ via carbamyl phosphate (Reichard - 1954).

In the absence of NH_4^+ or nucleic acid derivatives, the deamination of amino acids could serve as a source of NH_4^+ in the synthesis of GMP and pyrimidines.

The existence of glutamic acid dehydrogenase in Micrococcus sodonensis was clearly demonstrated by the microdiffusion technique. In the absence of an energy source, fresh resting cells of M. sodonensis deaminated glutamic acid and employed the resulting α-keto-glutarate as an energy source. The free NH_4^+ formed diffused into the medium. The addition of NH_4^+ to the medium did not alter this result. The addition of lactic acid as an energy source reduced the evolution of NH_4^+ to zero by relieving the cell of the need of employing glutamic acid as an energy source.

It should be stressed that glutamic acid dehydrogenase activity was determined in fresh, resting cells. NH_4^+ had no stimulatory effect upon resting cells. The stimulatory effect of NH_4^+ is restricted to actively growing cells. Therefore, the stimulatory effect must be associated with an activity which is more significant in growing cells - an activity such as nucleic acid or protein synthesis.

The determination of XMP aminase activity demonstrated a requirement for NH_4^+ in the formation of GMP in Micrococcus sodonensis. The amount formed by the deamination of glutamic acid was only 48% of that formed with NH_4^+. The enzyme control was assumed to be the base value. In the presence of salicylic acid, an inhibitor of glutamate dehydrogenase, no GMP was formed using glutamic acid as the amino donor. When salicylic acid was present with NH_4^+ and glutamic acid, an intermediate value was obtained. The possibility exists that the inhibitory effect of salicylic acid may not be limited solely to glutamate dehydrogenase. This would account for the lower value obtained.

The need of this enzyme system for NH_4^+ has been clearly demonstrated. In the absence of NH_4^+, the ability of the organism to deaminate glutamic acid was noted. The lower production of GMP in this case appears to be significant, and can be related to the increased growth response of the organism to exogenous NH_4^+.

The synthesis of carbamyl phosphate and its entry into pyrimidine synthesis appears to be the second major route of NH_4^+ incorporation in Micrococcus sodonensis. The actual synthesis of CAP was never demonstrated, but the reverse reaction, with the subsequent evolution of ATP, was determined. Reverse reactions are seen in many enzyme systems and the ability to shift the equilibrium of the system in this direction is useful in many biochemical studies.

Difficulty with the CAP synthetase system has been encountered elsewhere. Novoa (1964) found the instability

of CAP synthetase in animal cells to be caused by an inter-dependent reaction of acetyl-glutamate and cystine. The oxidation of cysteine to cystine resulted in a reaction with the sulfhydryl groups of the enzyme and consequent inactivation. The reaction was represented by the following:

$$Cy\text{-}S\text{-}S\text{-}Cy + E\text{-}SH \longrightarrow Cy\text{-}SH + E\text{-}S\text{-}S\text{-}Cy$$

The enzyme was calculated to have approximately 27 sulfhydryl groups per mole. These groups are needed to maintain stability of the enzyme. To prevent the oxidation of cysteine, cyanide was added. In this investigation, 7 μM of KCN and 5 μM of cysteine were added to each ml. of cell-free inoculum, but no difference was detected.

Jones (1963) describes a two-step reaction in CAP synthesis in bacteria. The first involves the formation of ammonium carbamate by means of a carbamyl kinase and the second step involves a carbamyl phosphokinase. This has been demonstrated in several species of Streptococcus and Bacillus.

The first step may be spontaneous according to Jones and Lipmann (1960), but the phosphokinase has been purified by Ravel et al (1961). Coupling the production of CAP with the formation of citrulline, by means of ornithine, realized an increase in CAP production of almost 2 fold. The Archibald method of citrulline analysis (1944) was used to measure the activity of the phosphokinase. When ammonium carbamate and ATP were employed as the substrates, 6.5 μM of citrulline

were produced. When ammonium sulphate and sodium carbonate-bicarbonate were used in place of carbamate, 3.5 μM of citrulline were produced.

Although a lesser amount of CAP is produced more slowly in the presence of carbonate, the authors do not exclude the possibility that the enzyme uses NH_4^+ and carbonate directly in the formation of CAP. The synthesis was reversible and an equivalent amount of ATP was formed.

The situation in animal cells is somewhat different. A single enzyme, carbamyl phosphate synthetase, produces one mole of CAP when two moles of ATP, and N-acetyl glutamate as a co-factor, are present. The reaction is essentially irreversible, and it has been suggested that this is a mechanism to overcome the unavailability of NH_4^+ in animal cells.

The synthesis of carbamyl phosphate in Micrococcus sodonensis was demonstrated to be completely reversible and this eliminates the possibility of the two-step mechanism found in animal cells by Fahien and Cohen (1963). Ravel et al demonstrated carbamyl phosphate synthesis from carbonate and NH_4^+ but to a lesser extent than with carbamate as the substrate. In the present investigation, carbamate was not employed as a substrate since the essential concern of the project was the incorporation of NH_4^+. The existence of the mechanism was demonstrated by its reverse reaction and the formation of carbamyl-aspartic acid, the most important product of CAP, insofar as this investigation was concerned, was ascertained.

The amino acid pool of bacteria is described by Britten and McClure (1962) as the total quantity of low molecular weight compounds that may be extracted from the cell under conditions such that the macromolecules are not degraded into low molecular weight subunits. Bacteria maintain internally-synthesized small molecules at high internal concentrations, and in addition, have the capacity to concentrate many compounds from the environment.

The present investigation was not concerned with the amino acid pool, other than its implications in the synthesis of nucleotides. Its obvious implication in the synthesis of protein was felt to be unrelated to the effect of NH_4^+ upon the metabolism of Micrococcus sodonensis.

The large amount of glutamic acid in the pool was not unexpected since this was the sole amino acid present in the synthetic medium. Aspartic acid, which was also present in the pool in high amounts, would be the result of glutamic acid transamination to oxaloacetic acid and/or the reverse product of the aspartase system. The enzymes of the Kreb's cycle have been demonstrated in Micrococcus sodonensis by Perry and Evans (1960), and the incorporation of oxaloacetic and fumaric acids into the cycle were observed. The significance of the aspartase system as a biosynthetic pathway has been suggested by Umbarger and Davis (1962) to be of little importance. Amination of oxaloacetic acid by glutamic acid transaminase has been demonstrated in detail by Kun et al (1964) and this possibility occurs in M. sodonensis. Amination of oxaloacetic acid by NH_4^+ would imply a dehydrogenase

system and this has been demonstrated only in the formation of glutamic acid and alanine.

The major roles of glutamic and aspartic acids in the biosynthesis of protein has been seen in many types of cells. Aspartic acid functions in the synthesis of purines and pyrimidines. Both aspartic acid and glutamic acid serve as precursors to other amino acids.

Glutamic acid leads to the formation of glutamine, proline, and ornithine which was present in appreciable quantity in the pool of M. sodonensis. Citrulline is formed by the carbamylation of ornithine, via carbamyl phosphate, and this synthesis leads to the formation of arginine. Although the synthesis of citrulline was demonstrated in a cell-free suspension of M. sodonensis, it was not found in the pool. It is probably used immediately and not stored appreciably.

Aspartic acid is involved in the formation of asparagine, threonine, methionine, lysine and isoleucine. The most interesting aspect of this investigation is its involvement in the synthesis of purines and pyrimidines.

In the synthesis of purines, aspartic acid was found by Buchanan (1960) and his co-workers to contribute the N_1 of the purine ring as well as the amino group of C_6 of adenylic acid.

The carbamylation of aspartic acid, demonstrated by Reichard (1954) in rat kidney tissue, is a key intermediate in the formation of pyrimidines. This system was demonstrated in M. sodonensis and is the second primary requirement of NH_4^+ in this organism. In the absence of NH_4^+, pyrimidines added to the medium functioned as well as NH_4^+.

Separation of the nucleotide pool of Micrococcus sodonensis showed it to contain the 5' monophosphates of the four ribonucleotides. Although the relative percentages of the nucleotides extracted by means of cold TCA were not calculated on a molar basis, the relative percentages of their optical densities were close to that found by Leitzmann and Bernlohr (1965) in B. licheniformis.

Entry of the heavy nitrogen isotope into the nucleotide pool is immediate and an equilibrium is established within 30 seconds. Entry into the amino acid pool is slower and an equilibrium is established much more slowly. Enrichment in the amino acid pool is probably, to a large extent, the result of exchange between amino groups of amino acids and free $N^{15}H_4^+$ within the cell.

The rapid equilibration in the nucleotide pool is indicative of an immediate need of NH_4^+ in the pool. Any N^{15} in the pool would initially be present in the N_1 of uridine and cytidine and the amino group of C_2 in the purine ring of guanosine. Eventually, transamination of the heavy isotope to aspartic acid would result in a higher level of the enrichment in the pool, since aspartic acid contributes its nitrogen to all four nucleotides.

The enrichment of each nucleotide could be demonstrated by cold TCA extraction of a large culture grown in an N^{15}-enriched medium. After separation of the components of the extract by ion exchange chromatography, the degree of enrichment of each component could be determined by mass spectrometric analysis. The unequal proportions of nucleotides in

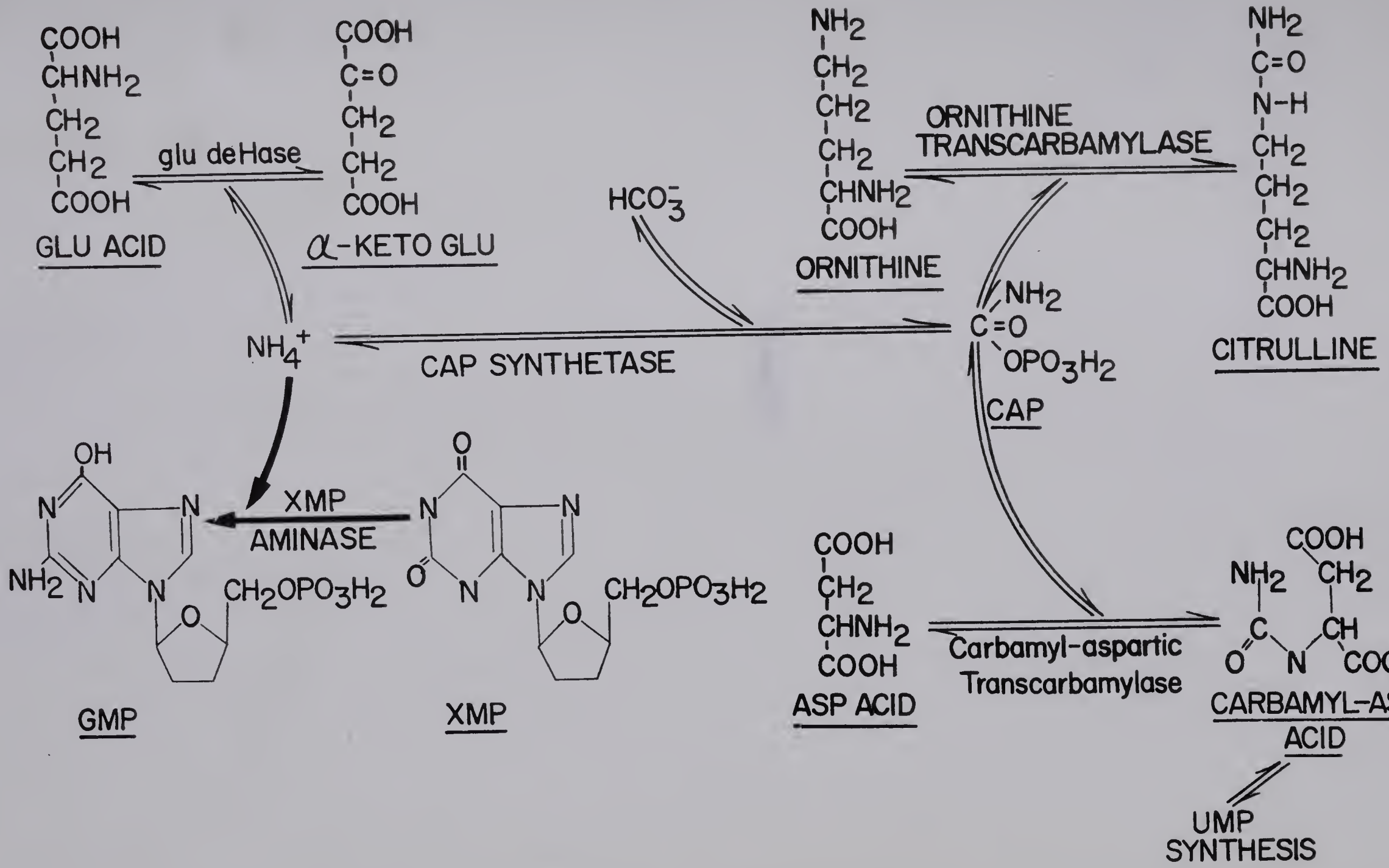
the pool, however, would present a problem in sample size - one mg. of nitrogen being necessary for an accurate analysis. The extended procedure for sample preparation would lead to further sources of contamination which would have to be overcome.

Radioactive studies would be less of a problem, insofar as sample size is concerned. An interesting possibility would be the addition of C^{14}-labelled carbonate, xanthosine and aspartic acid to the medium. The rate of the label's appearance in the nucleotide pool would be determined in the presence and absence of NH_4^+. The components would be separated by paper chromatography and the spots measured for radioactivity.

Metabolic Chart

of

Ammonium Pathways



METABOLIC PATHWAYS OF NH_4^+

APPENDIX

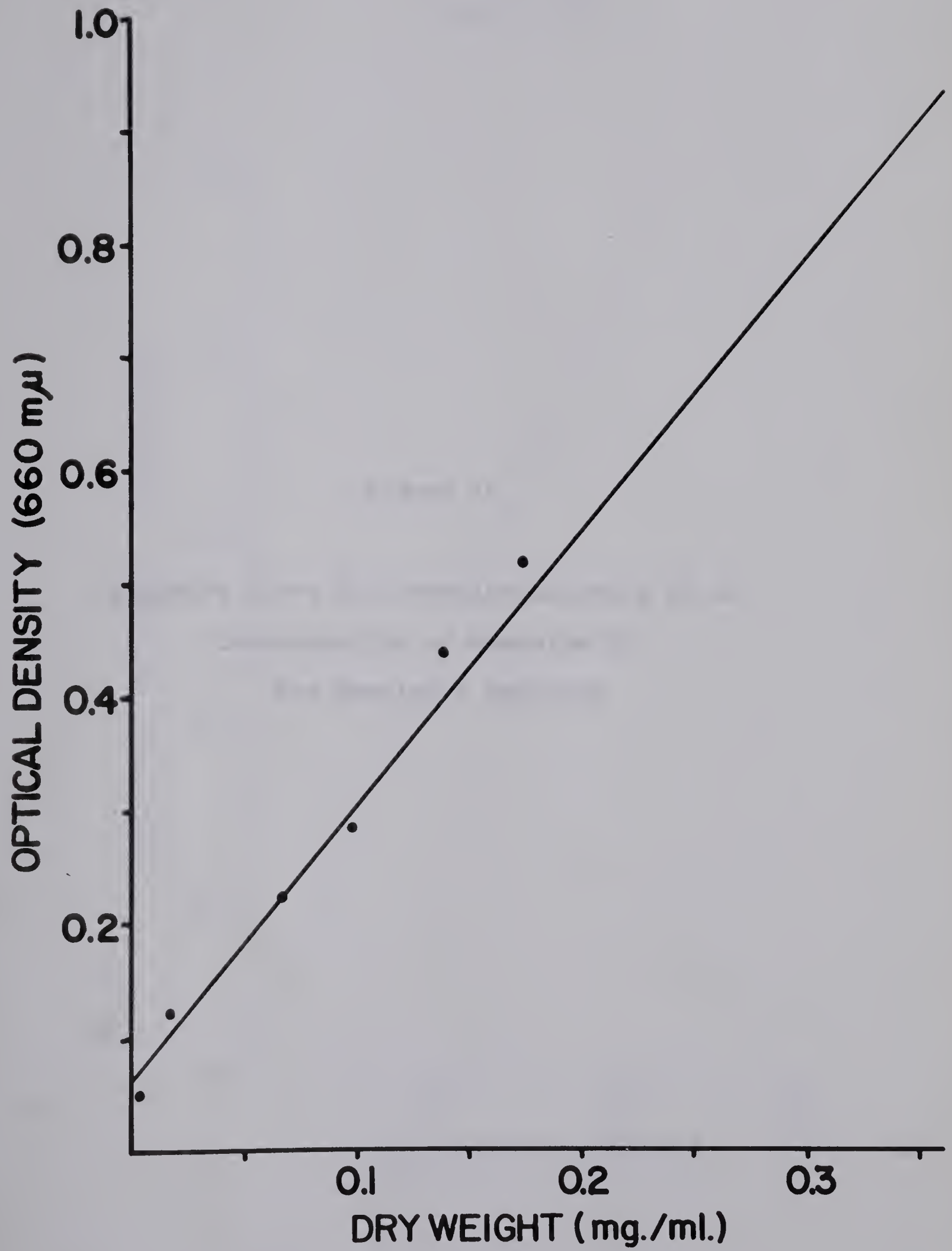
Veronal Buffer

diethyl barbituric acid	8.712 g./l.
NaOH	1.893 g./l.
$NaOCOCH_3$	6.476 g./l.
HCl (0.1M)	60.0 ml./l.

Figure 14

Standard Curve for Suspensions of Washed Cells of M. sodonensis Relating Optical Density to Dry Weight of Cells

FIGURE 14



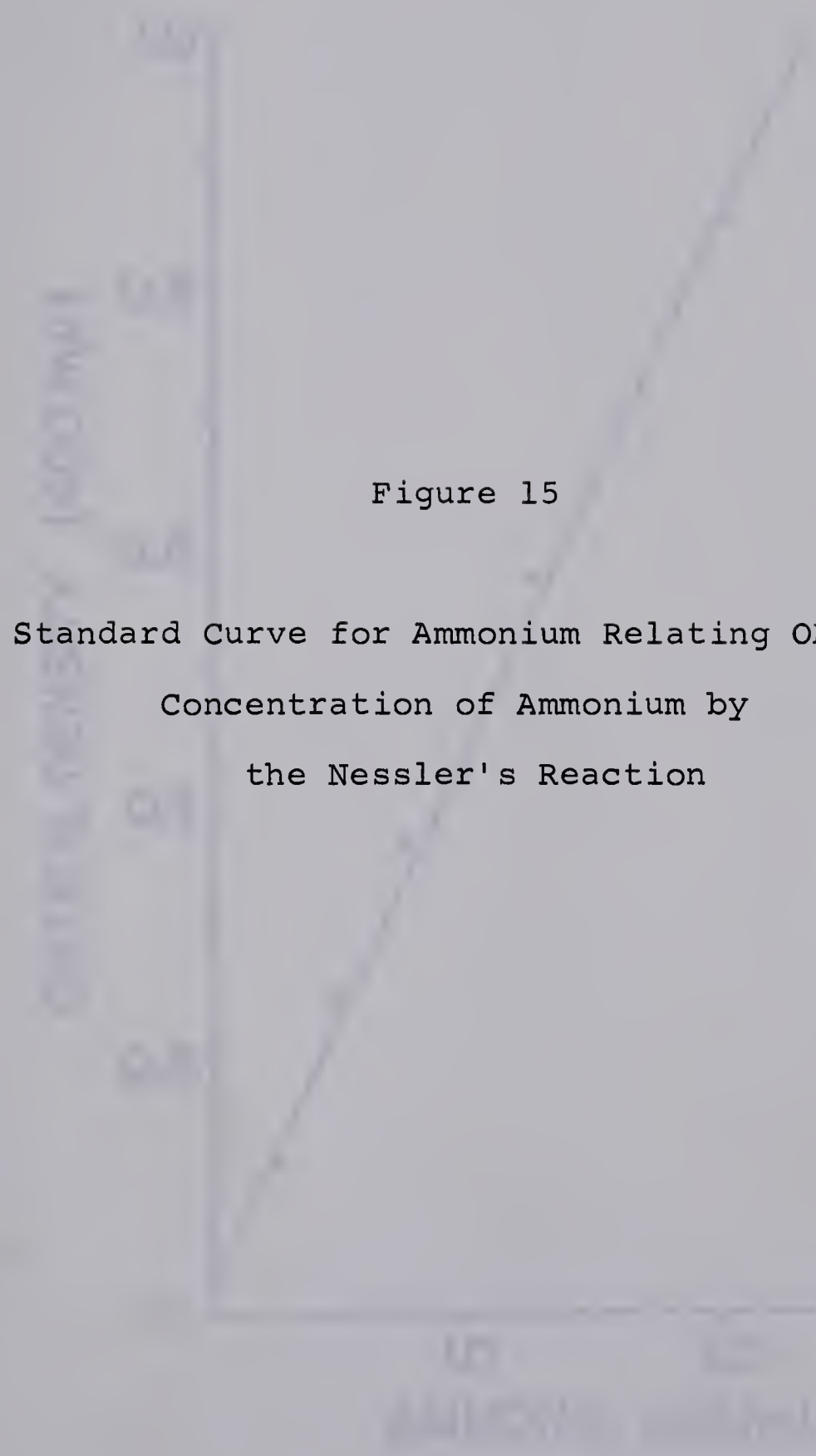
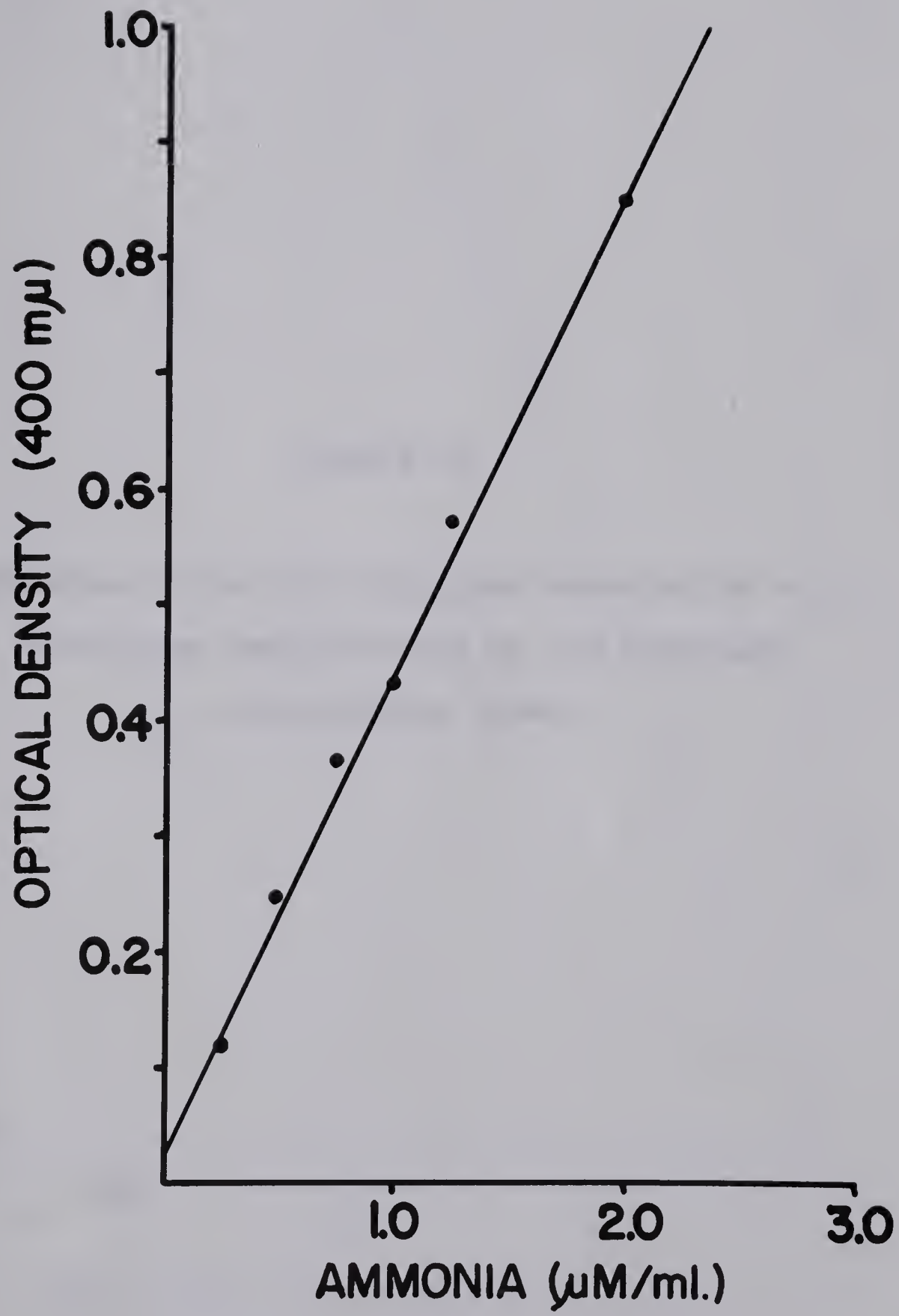


Figure 15

Standard Curve for Ammonium Relating OD to Concentration of Ammonium by the Nessler's Reaction

FIGURE 15



1.0
0.8
0.6
0.4
0.2
ORTHOGRAPHIC VTRE (μm)

Figure 16

Standard Curve for Citrulline Relating OD to Citrulline Concentration by the Technique of Archibald (1944)

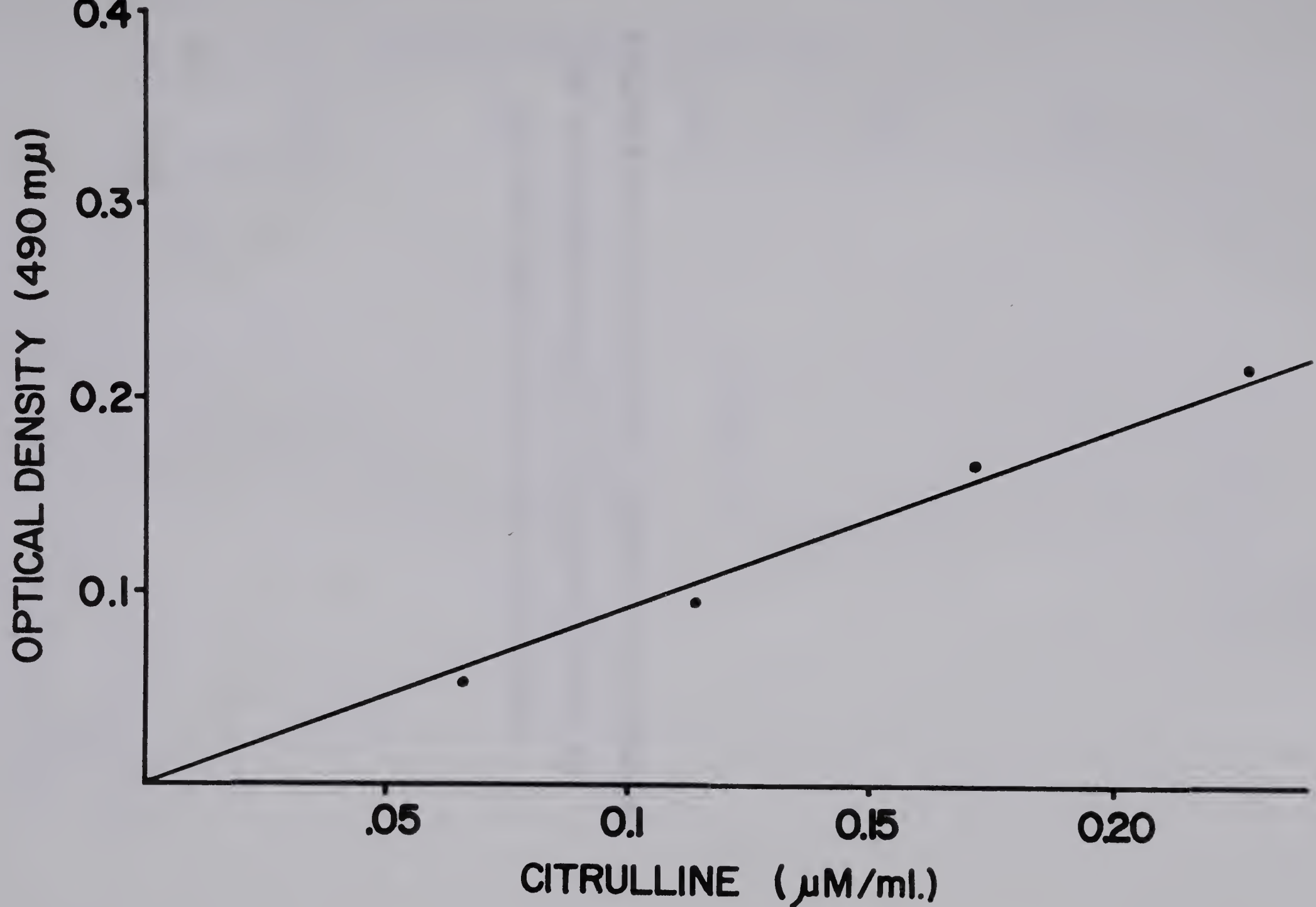
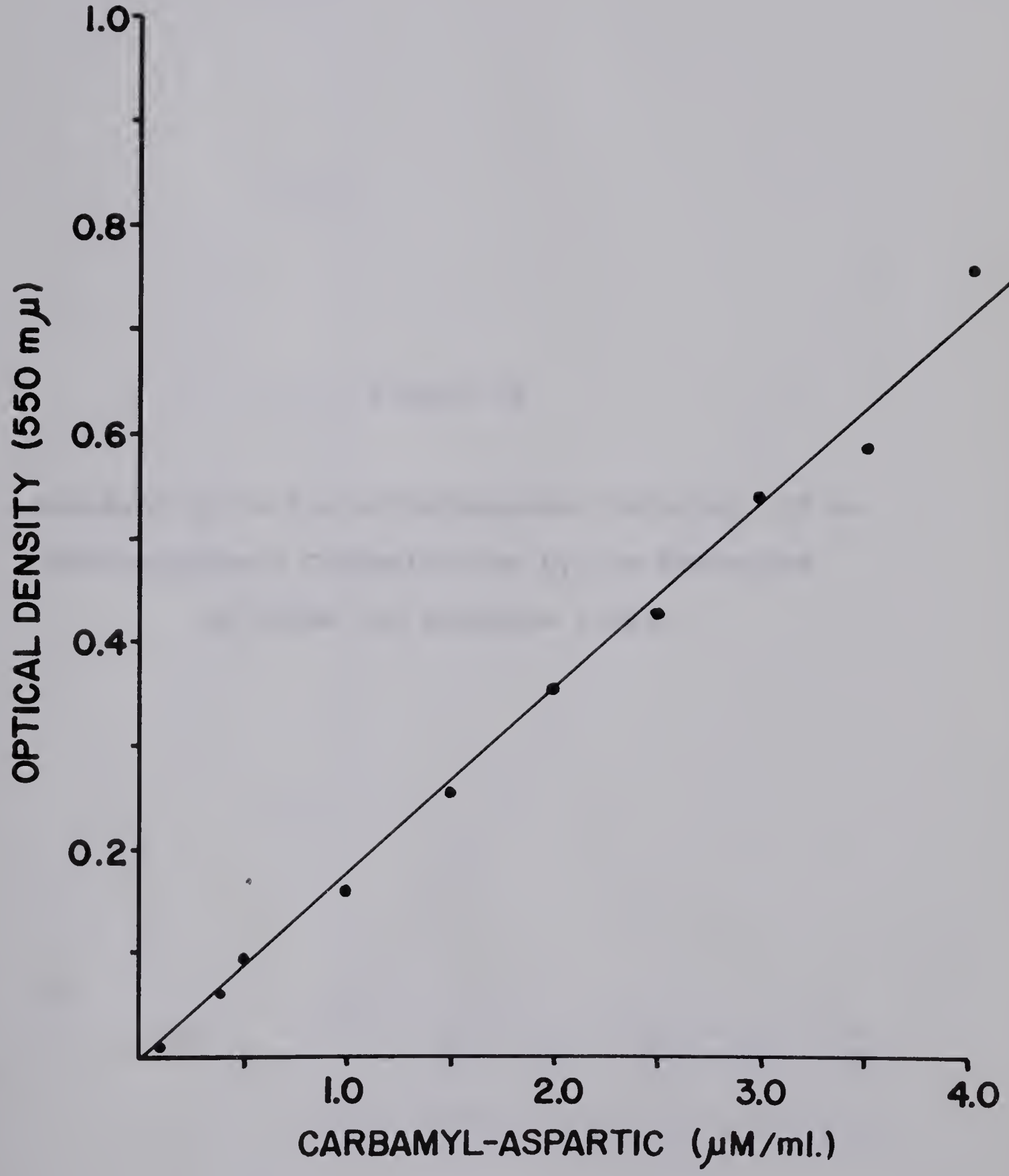

OPTICAL DENSITY (490 mμ)
0.4
0.3
0.2
0.1
.05
0.1
0.15
0.20
CITRULLINE (μM/ml.)

FIGURE 16

Figure 17

Standard Curve for Carbamyl-Aspartic Acid Relating OD to Carbamyl-Aspartic Acid Concentration by the Technique of Koritz and Cohen (1954)

FIGURE 17


1.0
0.8
0.6
0.4
0.2
OPTICAL DENSITY (550 mμ)
1.0
2.0
3.0
4.0
CARBAMYL-ASPARTIC (μM/ml.)

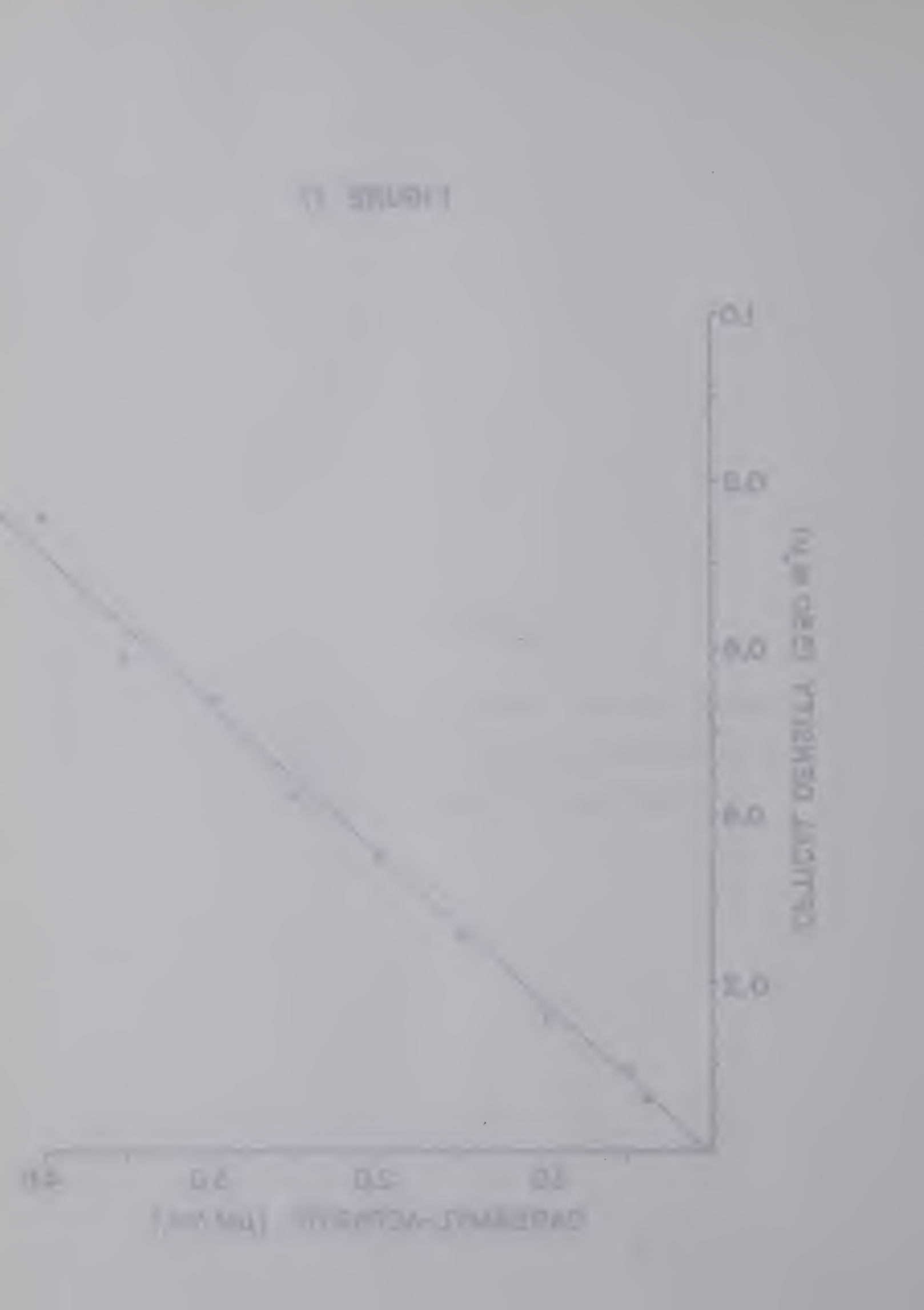
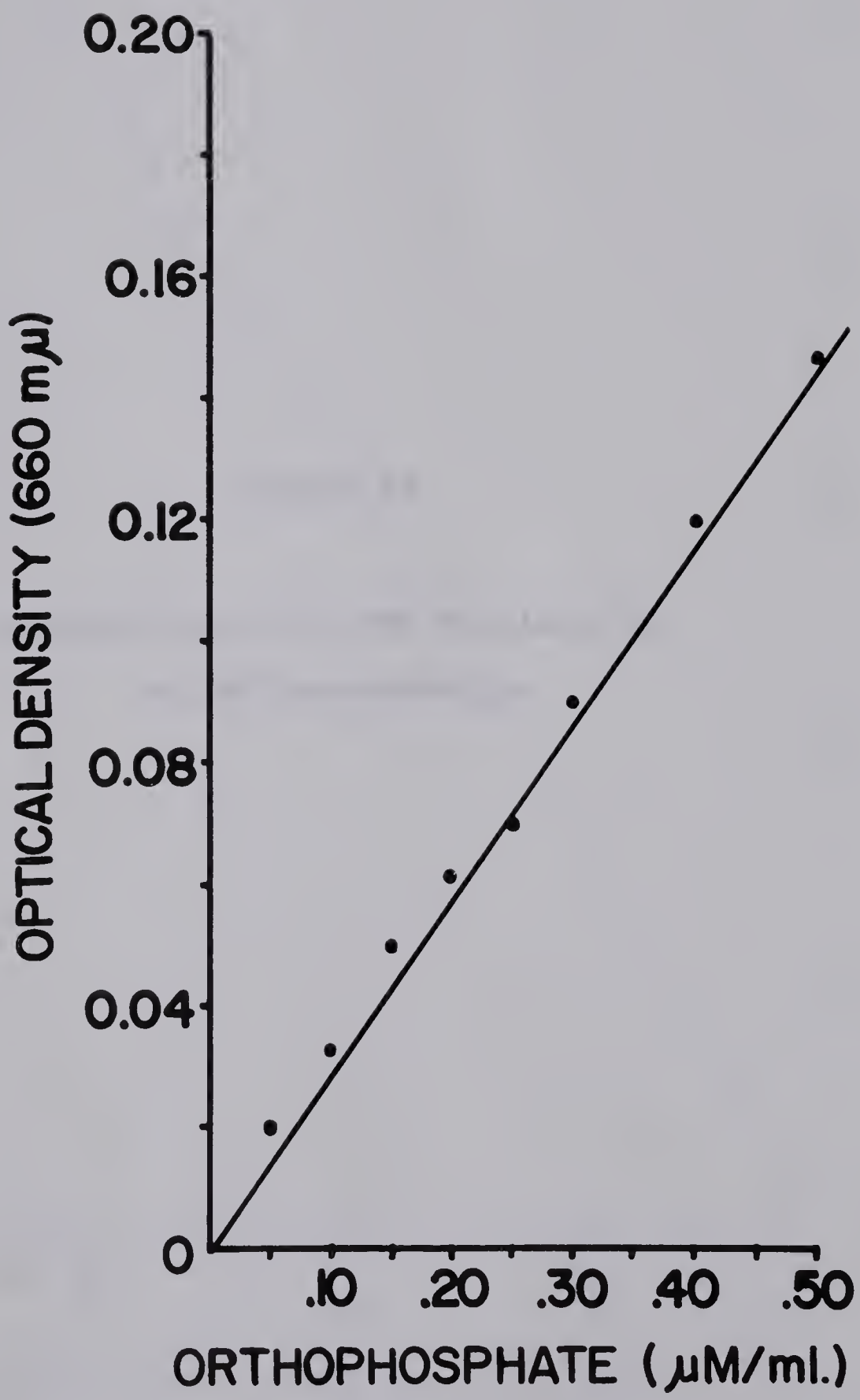


Figure 18

Standard Curve for Orthophosphate Relating OD to Orthophosphate Concentration by the Technique of Fiske and SubbaRow (1925)

FIGURE 18


0.20
0.16
0.12
0.08
0.04
0
OPTICAL DENSITY (660 mμ)
.10
.20
.30
.40
.50
ORTHOPHOSPHATE (μM/ml.)


0
0·05
0·10
0·15
0·20
0·50

Figure 19

Standard Curve for GMP Relating OD

to GMP Concentration

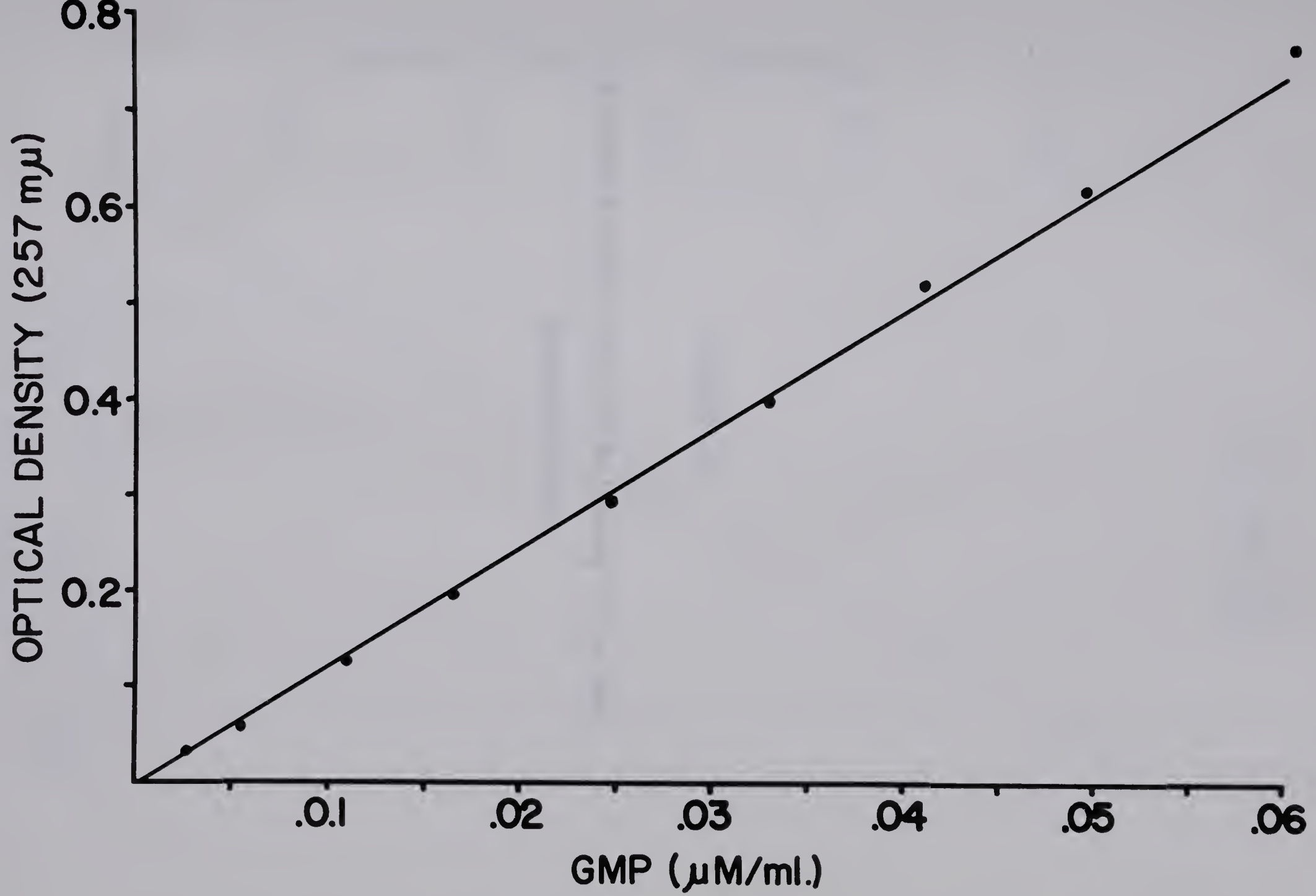


FIGURE 19

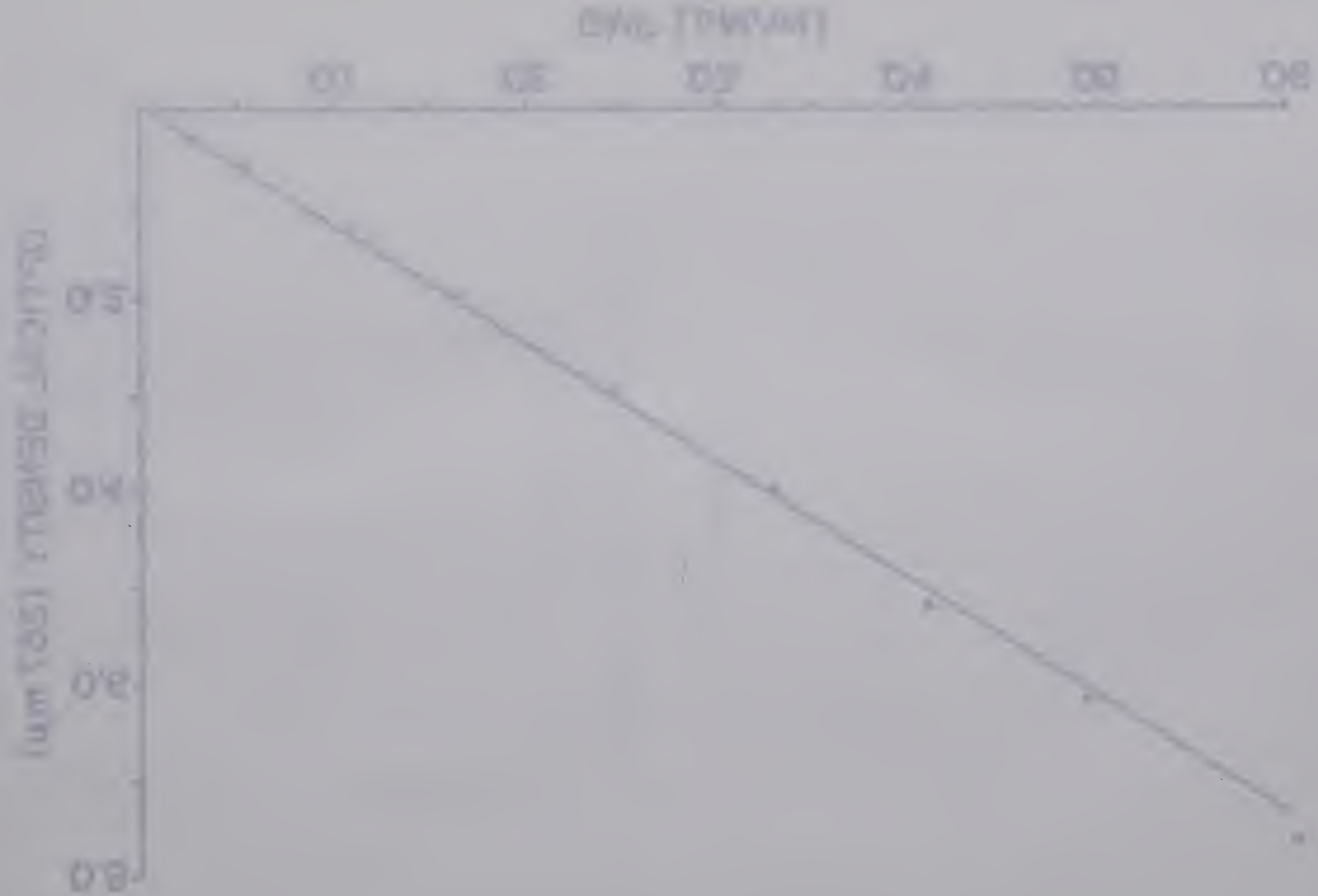
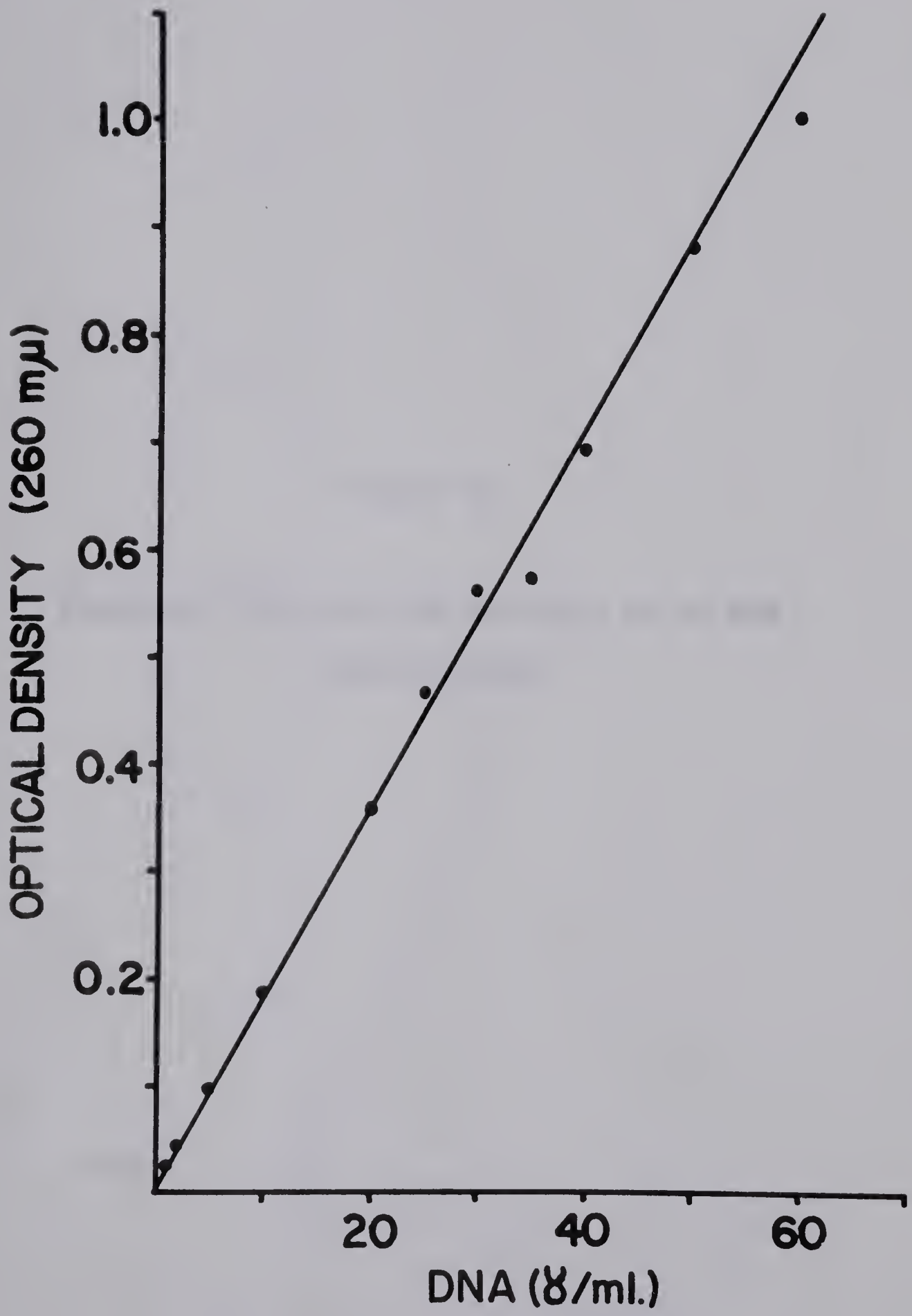


Figure 20

Standard Curve for DNA Relating OD to DNA Concentration

FIGURE 20

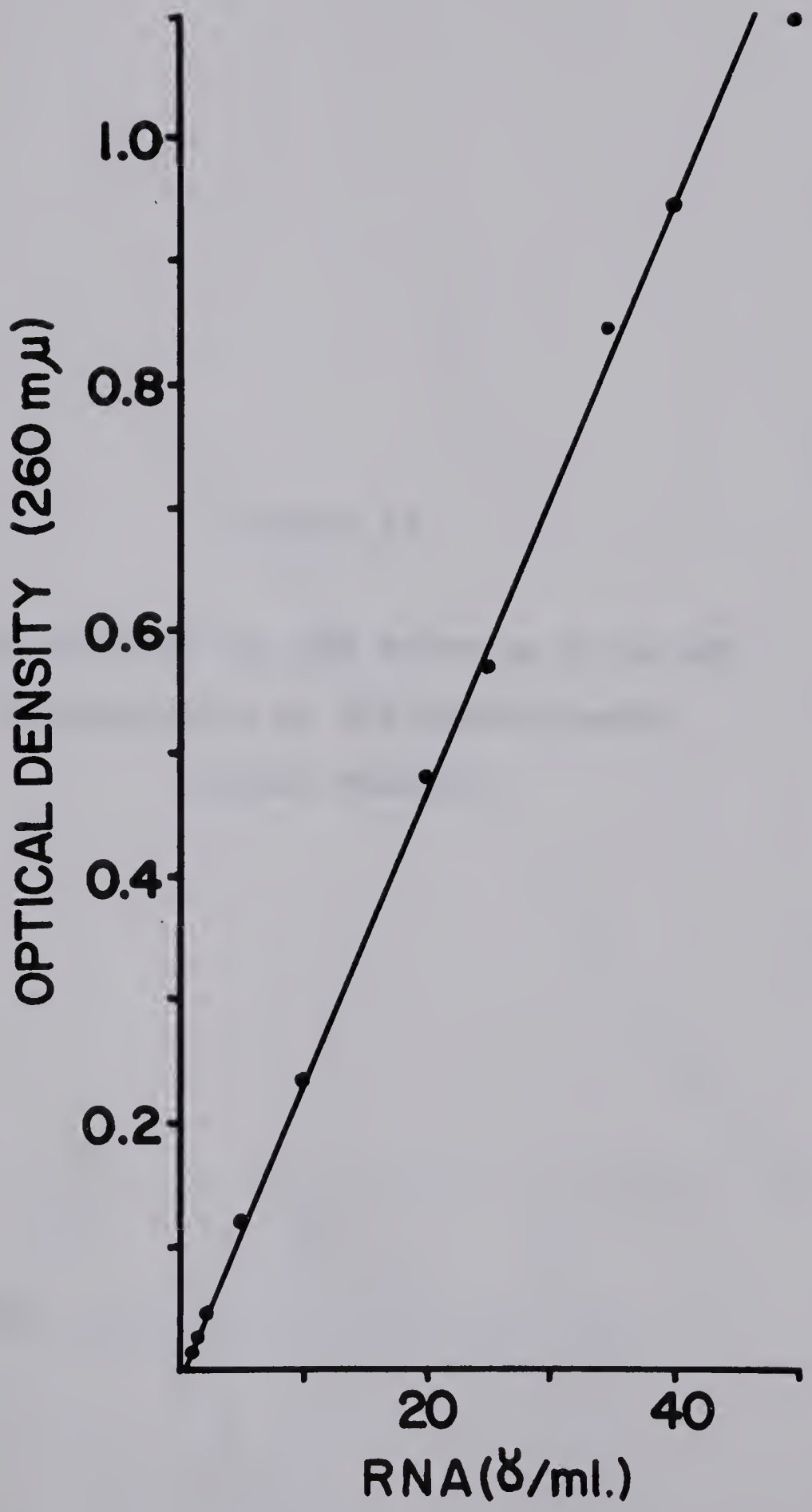


1.0
0.8
0.6
0.4
0.2

Figure 21

Standard Curve for RNA Relating OD to RNA Concentration

FIGURE 21



1.0
0.8
0.4
0.2

Figure 22

Standard Curve for DNA Relating OD to DNA Concentration by the Diphenylamine Colour Reaction

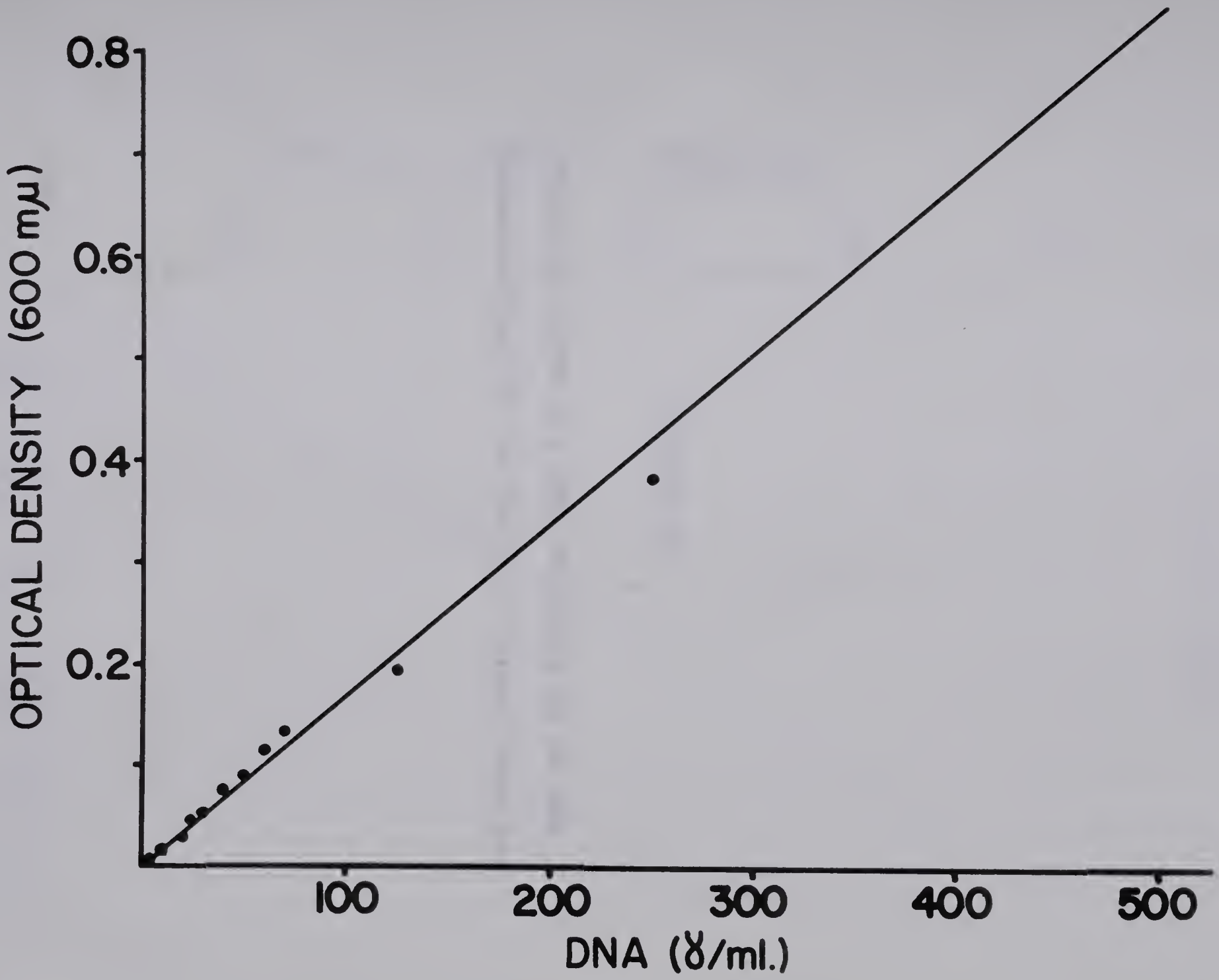
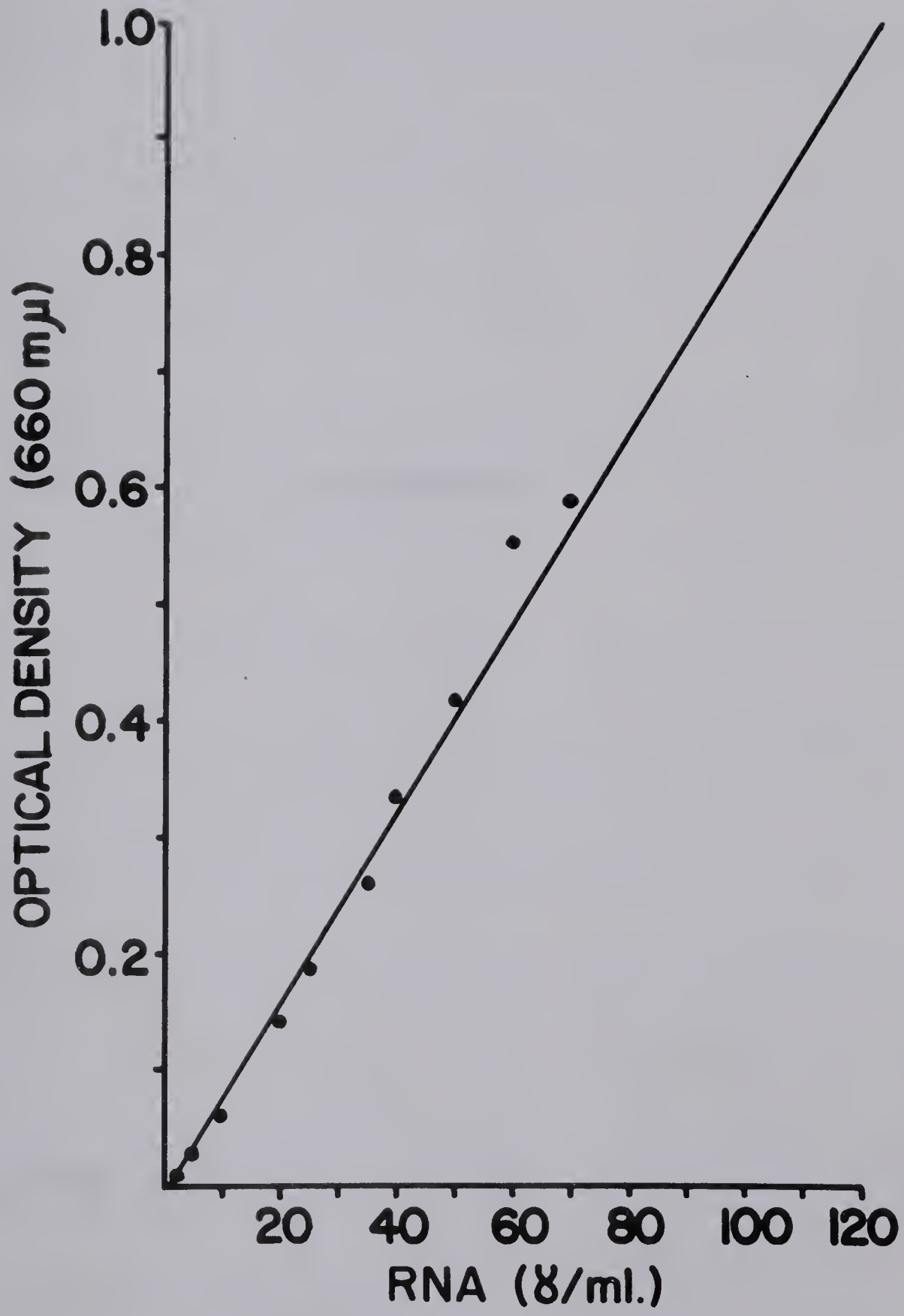


FIGURE 22

Figure 23

Standard Curve for RNA Relating OD to RNA Concentration by the Orcinol Colour Reaction

FIGURE 23



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